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ABSTRACT

This guidebook provides information related to developing laboratories for an introductory college-level course in mycology. This information will enable mycology instructors to include information on less-familiar organisms, to diversify their courses by introducing aspects of fungi other than the more strictly taxonomic and morphologic, and to receive guidance on fungi as experimental organisms. The text is organized into four parts: (1) general information; (2) taxonomic groups; (3) ecological groups; and (4) fungi as biological tools. Data and suggestions are given for using fungi in discussing genetics, ecology, physiology, and other areas of biology. A list of mycological films is included. (Author/SA)

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Mycology Guidebook

Mycology Guidebook Committee, Mycological Society of America

Russell B. Stevens, Editor

U.S. DEPARTMENT OF HEALTH,
EDUCATION & WELFARE
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EDUCATION

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Preface

Mycology, as commonly taught at the introductory level to undergraduates and beginning graduate students, stresses classical morphology and taxonomy and relies much on the use of preserved specimens. This practice has certain advantages in terms of convenience and it would be pointless to argue that present instruction is without merit. On the other hand much can be done to improve the presentation of fungi in at least two directions: (1) Introduction of considerably more living material and (2) supplementation of the orthodox morphologic view with information from genetics, physiology, industrial mycology, fungus ecology and medical mycology. Unless changes of this nature can be effected, mycology will continue to present less than its full range of interest to the student.

This *Guidebook* is intended to facilitate improvements in introductory mycology teaching. It is neither a laboratory manual for the student nor a compendium of laboratory exercises in the usual sense of that term, but assembles information to which the teacher may turn for direct assistance in developing the laboratory aspects of his own course. While instructors in other courses and at other levels can find much of value in the *Guidebook*, principal stress is on college courses in mycology at the introductory level.

Specifically, the *Guidebook* attempts to provide useful answers to three questions: (1) How to obtain desired mycological materials, especially by field collections in diverse ecological situations; (2) how to maintain fungus specimens in the laboratory in such fashion that they will be dependably available when needed in the teaching program; and (3) how to present selected groups of the fungi in their most provocative, preferably living, condition. Additionally, there are data and suggestions on how best to use fungi effectively in elucidating genetics, ecology, physiology and related biological specialties. The key objective, in sum, is not drastically to alter mycology courses but to provide resources whereby instructors may substantially improve them.

Five more or less distinct kinds of information are available to the user of the *Guidebook*, although it will be clear from the outset that a certain amount of overlap appears from one category to the next:

PART I. General information, including those suggestions on field collecting, isolation techniques, maintenance of cultures and specimens, and related matters that are applicable to a variety of situations and for a substantial number of different kinds of fungi.

PART II. Taxonomic Groups. For each of a considerable array of commonly taught natural groups of fungi, this section offers specific data on where desired material may be sought, how it may be maintained, prepared for and presented in class, and in what references more detailed data are available. This section is by no means encyclopedic, nor does it dwell on problems of fine taxonomic distinction. Commonly used names are employed as a handy device for labelling the separate sections, but there is no pretense to a definitive taxonomic treatment.

PART III. Ecological Groups. For some fungi, or groups of fungi, greatest interest lies in the fact that they occur in distinct ecological situations, are associated with other organisms in a more or less unique way, or exhibit one or more particularly striking morphologic or physiologic characteristics. Whether they are or are not taxonomically closely related is of secondary interest. Part III contains information of this general nature, to be used from time to time in conjunction with the more taxonomically oriented materials in Part II. That is, Part III emphasizes those fungi or groups of fungi that are of first interest because of where they are found or what they do rather than because they represent significant segments of the taxonomic scheme.

PART IV. Fungi as Biological Tools. Certain biological phenomena are, for one reason or another, particularly well demonstrated by using mycological materials. This section of the *Guidebook* contains several specific and detailed suggestions as to how these demonstrations may be carried out, with information on sources of fungi to be used and techniques by which they can be successfully handled. It contains, also, several items best designated simply as "special materials."

Literature citations and information on culture repositories, on stains and media, and on available films are provided as appendices. There is also an alphabetical list of fungi that provides page references for each instance wherein a given organism is treated in the body of the *Guidebook*.

The *Guidebook* should be useful in several ways. For the instructor who seeks to improve his treatment of the groups of fungi commonly encountered in the introductory course, Part II is designed to provide useful information. It is particularly aimed at encouraging those teachers who may have fallen into the practice of emphasizing groups with which they are familiar to branch out and to include less well-known forms--and especially to assure that such new exercises as they may introduce are a success by providing them with the advice of specialists.

A second use of the *Guidebook* is to encourage mycology teachers to diversify their courses by introducing aspects of fungi other than the more strictly taxonomic and morphologic. Thus Part III will be especially useful to the instructor who wishes to retain the traditional approach to mycology but to show his students, at an early stage in their training, that mycology offers a very diverse field of endeavor. Part III will best be used, probably, as a means to enliven an introductory course that has already been rounded out with the help of suggestions in Part II.

Thirdly, the *Guidebook* is planned to be of use to biologists in general by providing guidance on fungi as experimental organisms. This is not necessarily to suggest that mycologists themselves should avoid the operations summarized in Part IV, but rather that they not necessarily be confined to mycology courses. Not infrequently, perhaps, fungi provide material that more dramatically illustrates a given biological phenomenon than does the material traditionally used.

Lastly, certain more complex material is included that may be incorporated into advanced courses. This feature is not a major emphasis of the book but should make it somewhat more widely useful.

The *Guidebook* results from the invaluable contributions of a host of individuals. We have not attempted to preserve the identity of each separate piece, but rather to meld the entire accumulation of data and opinion into a coherent whole. To the best of our ability, the name of every person who has been called upon to provide scientific material is listed in the final Appendix, and we are most happy to acknowledge our important reliance upon their assistance in compiling this volume.

It will be apparent that the preponderance of examples chosen for this compilation come from U. S. sources, although a number of important items were derived from our overseas colleagues. Generally, however, each of the procedures can fairly readily be adapted to the species available and the conditions prevailing in other localities.

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PART I

GENERAL INFORMATION

1 Handling Fungi

FIELD OBSERVATION OF FUNGI

One of the better methods of presenting the fungi to a class is to go to the field and let the students observe the different kinds that are available. Nearly all major groups of fungi can be observed in a trip to a convenient woodlot in season.

Within such a woodland, different groups of fungi can be observed on various substrates. On the ground there will be a variety of mushrooms; on the wood of an old fallen log, dead branches on the ground, stumps, etc., an assortment of bracket fungi and several types of Ascomycetes including small to medium-sized cups and effused black or red Pyrenomycetes. Since some of the cups belong to Basidiomycetes, however, microscopic examination of specimens in the laboratory is important. The underside of sticks, branches, and old logs may harbor effused resupinate species with smooth, warted, toothed, or porose hymenial or lower surfaces. Some of these various types of fruit bodies, especially if they are slightly old, or if they have dried and have been revived by summer or autumn rains, may be overgrown by several types of mold fungi and yeasts, not to mention bacteria that may become nuisances if attempts are made to culture the fungi.

In general, the best season for viewing fungi of many kinds is the autumn, especially after a few good rains.

After a dry summer there is usually a lag of 10 days to 2 weeks before autumn-fruiting species start to appear in sufficient numbers to make a field trip worthwhile, although this varies according to the region. One may well rely on the past experience of some colleague who has observed fungi in the field. In the Middle West a generally favorable time to observe fungi in the field is late summer to the end of October. In the Far West a good time to conduct field trips is about three weeks after the autumn rains start. In the southern and northwestern states early autumn should be a good season.

Since soil mycelia are usually perennial, certain species continue to produce fruit bodies year after year with regularity and in about the same numbers. Many species fruit regularly, but numbers of fruit bodies may be controlled by as yet unexplained variations in precipitation, humidity, and food supplies, within certain unsuspected or poorly understood genetic limits. In addition to those species that grow only on the decaying organic matter present, there is a series of fungi, especially mushrooms, that forms mycorrhizae or "fungus roots" with many of the woody plants (see p. 321). Some, if not all, of these species also use litter and humus in various stages of decomposition.

Some of the more common North American woodland habitats include pine and other conifer forests, pine-hardwood mixed forest, birch-aspen woods, beech-maple woods, oak-hickory woods, and soft-maple swamps. These are discussed under "Homobasidiomycetes" (p. 278). Certain other, special ecological niches, however, are worthy of mention here..

Burned Areas (Petersen, 1970) These support a group of fungi that are widespread but very rarely fruit in any other habitat. This "flora" consists largely of Ascomycetes belonging to the Pezizales. Many Discomycetes with very small apothecia occur in large quantities and are excellent for class work. Some agarics also occur in these areas. In some regions (central Idaho in particular) *Morchella conica* occurs in great abundance after fires, but since this fungus is also common outside of burned areas, it differs from the others at least to some degree, as far as fruiting habits are concerned. The stimulus for fruiting appears to be the release of mineral nutrients during the burning process; a change in the balance of nitrogen-containing radicals is also thought to contribute to this stimulus.

Sand Dunes *Laccaria trullisata* and species of *Hebeloma* and *Psathyrella*, as well as certain Gasteromycetes (see p. 295), are usually found in late summer in the Great Lakes region.

Manure The dung of various animals has long been recognized by teachers as an ideal substrate for producing class material and for studying fungus ecology. The substrate can be brought into the laboratory, placed in moist chambers, and watched closely for the fruiting of various species of Phycomycetes, Ascomycetes, and Basidiomycetes. Fleshy fungi, especially species of the Bolbitiaceae, Coprinaceae, and Strophariaceae, are often found on this substrate in nature and can be grown and fruited in the laboratory in pure culture on dung decoction agar. These forms are especially desirable as teaching aids because the basidiocarps are relatively delicate and go through their entire development in a matter of hours; the whole process can be followed conveniently in the laboratory. *Coprinus ephemerus* is a well known laboratory subject.

Manure produces a succession of fungi. Freshly dropped manure will not produce certain species that do appear on over-wintered manure, and vice versa; this represents a type of ecological specialization.

Moss Beds and Sphagnum Bogs This is a favorite habitat for agarics and Discomycetes with small sporocarps. From the standpoint of the Agaricales, moss beds can be divided into a dry and a wet type. In the dry type, such as beds of *Polytrichum piliferum*, in sandy areas we find agarics occurring in early spring before the habitat has dried out. Species of *Psilocybe* are most numerous and appear in April in the vicinity of Ann Arbor, Michigan. In mountain meadows they fruit shortly after the snow has melted.

The wet moss flora is relatively luxuriant but can be confused with the species of the terrestrial flora.

In *Sphagnum* bogs, *Lyophyllum palustre* appears to be parasitic on *Sphagnum*, as is evidenced by the patches of killed moss in the area on which the basidiocarps develop. In mid-spring to early summer, agarics belonging to the genera *Galerina* and *Clitocybe* are characteristic of *Sphagnum*. The *Clitocybe* basidiocarps arise directly from the upper part of living gametophytes.

Snow-bank Fungi In timberline areas of the western United States, a number of species of fungi are associated with organic matter. Some of these species fruit only when irrigated by snow-melt water. While soil temperatures may reach 0-3°C, enough warmth may be generated in the fruiting process to melt snow in the immediate vicinity of growing or developing mushrooms. It might be well to study such fungi in relation to fungal cryophily and to consider the use of such species in research on cold-temperature related processes. It might also be informative to compare the snow-bank with the permafrost habitats. (See also Cooke, 1955.)

Arid Regions The arid desert regions of our West and Southwest produce Gasteromycete desert genera such as *Montagnites*, *Podaxis*, *Gyrophragmium*, *Battarraea*, *Tulostoma*, etc., but some large *Calvatia* species occur in the sagebrush habitat.

COLLECTION, LABORATORY CARE, AND STORAGE

The collecting, laboratory care, and storage techniques described for "Homobasidiomycetes" (p. 278) are generally applicable to most fungi collected on a field trip. Lignicolous, nonfleshy fungi, which are relatively nonfragile, may be wrapped in newspaper, waxpaper, or placed in paper bags. Myxomycete specimens should be transported in individual small boxes that are kept upright, although some of the tougher species may be loosely wrapped in paper instead (see p. 41). Herbaceous plants containing leaf-inhabiting fungi, if in danger of drying so much as to curl up, may be carried back to the laboratory in large plastic bags or placed in an ordinary plant press in the field.

Further details on collecting special groups of fungi may be found in the sections dealing with the respective groups.

ISOLATION, OBSERVATION, AND CONSERVATION

To a great extent, "collection" of very small (as well as a number of very large) fungi has come to include isolation and cultivation of material. Successful isolation work takes advantage of time-honored tricks with hyphal tips and single spores as well as of growth rate differences, temperature and nutrient responses, and differential resistance to biostatic and biocidal agents. Techniques especially useful in handling certain taxonomic and ecologic groups are discussed in the treatments of those groups. Similarly, techniques of special value in the cultivation and preparation of isolates for microscope study are described in the material dealing with the fungus groups with which they are commonly used. For additional information, see Commonwealth Mycological Institute (1960), Savile (1962), and Booth (1971a).

However, enormous numbers of preparations for microscope study are made simply by mounting fungus elements from the original substrate or from cultures directly in a drop of lactic acid or of lactophenol (SR-1) and flattening the preparation under a cover slip before examination. An additional uncomplicated procedure, the slide culture technique, yields excellent preparations of those fungi that are capable of sporulating or producing other characteristic elements in culture.

General instructions for the slide culture technique, for the preparation of a kind of semipermanent slide mount useful for a wide range of organisms, and for maintaining stock cultures are outlined in the immediately following sections.

Slide Culture Technique

Growing a fungus as a slide culture will result in beautiful preparations in which the sporulation characteristics of the organism remain relatively unchanged.

This is accomplished by growing fungi on a block of agar sandwiched between a glass slide and a cover slip in a moist chamber (Figure 1).

- Prepare Petri dish with agar suitable for organism to be grown; use about 15 ml of agar per 100 mm dish. Cut solidified medium rapidly into 1 cm squares, using a sterile knife or dissecting needle and a sterile glass rod.
- Place a bent glass rod on a filter paper disc in the bottom of a Petri dish, add slide, cover and sterilize. Introduce sterile water just sufficient to moisten filter paper (with 5% glycerin if fogging occurs on slide).
- Place agar block on slide, using aseptic techniques throughout.
- Inoculate the center of each of the four sides of the agar block with spores or mycelium.
- Place a sterile cover slip centrally upon the agar block. Incubate under temperature and light conditions suitable to sporulation of the organisms. (Add more sterile water if filter paper dries.)
- Lift cover slip carefully; discard agar block.
- Place drop of lactophenol on a clean slide and add the cover slip with the fungus growth adhering to it.

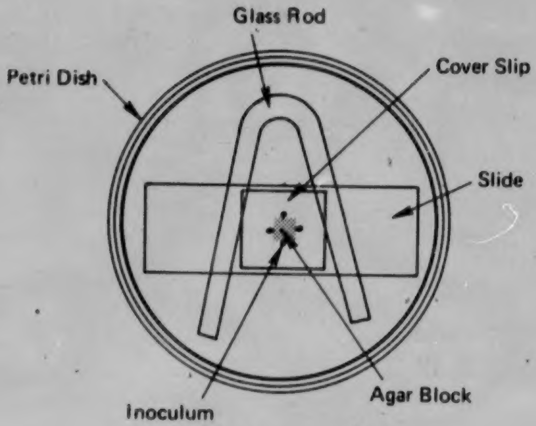

FIGURE 1 *Diagram of a moist chamber.*

Place a drop of lactophenol in the center of the fungus growth on the culture slide; cover with a clean cover slip.

Permanent Slide Mounts

Microscope slide mounts that will remain usable over a period of years are a necessity in many mycological teaching and research situations. Preparations in the commonly used lactophenol (SR-1) can be protected if basic precautions are taken and if a cement that will not react with the liquid mounting is used.

- Work with scrupulously cleaned slides and cover slips. (Dusty or fingerprinted glass is the first step toward a poor seal.)
- Use the smallest cover slip consistent with the dimensions of material being mounted. (A remarkably high percentage of materials can be handled easily under 13 mm square cover slips. Cover slips larger than 18 mm square or in diameter should be avoided, since they reduce the slide marginal area available for application of two or three layers of sealant.)
- Place a small drop of lactophenol, lactic acid, or other suitable mounting medium on the slide. (Purely aqueous mountants are unsuitable for sealing; the water ordinarily used in making lactophenol may be omitted unless dyes soluble in water but not in the other ingredients are being used.) Learn to use an amount of liquid just sufficient to fill the space under the cover slip; a definite aid is the use of pipettes that have been drawn with a finer aperture than is found in most dropping-bottle pipettes.
- Mount and arrange specimen as desired. If fungus elements tend to shatter or to entrap air bubbles when placed in mounting fluid, begin a new slide preparation in a drop of alcohol or ethyl acetate; when the wetting agent has almost evaporated, add the required drop of lactophenol and continue.
- Lower cover slip onto preparation carefully, and at an angle, in order to avoid entrapment of air. (Prevent spores and other floating elements from moving to edge of cover slip by making original mount in a very small

drop that is flanked on left and right by separate short streaks of the mounting fluid; movement of the liquid streaks under the weight of the cover slip will concentrate floating elements in the center of the preparation.)

- Warm the slide gently in order to expel air bubbles, to swell and clear fungus elements, to concentrate stains, and to evaporate moisture. Do not boil.
- If a considerable amount of lactophenol has run from beneath the cover slip, the excess must be removed before sealing; filter paper or paper tissues applied carefully to the edge of the mount are suitable absorbers. (If the liquid does not quite fill all space under edge of cover slip, resist the temptation to add more mountant, since sealing material will fill in.)
- Apply a thin line of colorless nail polish (Note #1, below) or ZUT (Note #2, below) to join all edges of the cover slip to the slide. Allow to harden. If excess lactophenol had to be removed earlier (in which case the initial seal will be imperfect and probably will not harden completely), wash the mount in a gentle stream of water until all surface mountant outside the seal has been removed. Finish in distilled water. Set aside to dry thoroughly.
- Apply an additional thin layer of sealant, covering edges of previous layer. Allow to dry. Finish with one or two heavier and broader layers (not necessarily colorless, after the first), always allowing sufficient time for hardening between applications.

Note #1--Not all brands of nail polish are suitable; experiment with a few until one is found that adheres well to glass without chipping or cracking.

Note #2--A commercial preparation known as ZUT (Bennett's Slide Ringing Compound) has proved superior to nail polish in several laboratories and can be recommended. It thins satisfactorily with most lacquer thinners and most esters and ketones (e.g., n-butyl acetate), forms a strong seal without mixing with lactophenol, and retains a slight but desirable degree of plasticity after the solvent evaporates. Only source is the manufacturer: Bennett's Paint Products, 65 West First South, Salt Lake City, Utah, 84110.

Note #3--Additional hints and suggestions on the use of other mounting and sealing preparations may be found herein on p. 195 (permanent slides of Laboulbeniales); in the I.M.I. Handbook (Commonwealth Mycological Institute, 1960); in C.M.I. Plant Pathologist's Pocketbook (Commonwealth Mycological Institute, 1968); and in Dring (1971).

Stock Culture Maintenance

A balance must be struck between the periodic necessity of transferring stocks and the occasional loss of an isolate. The following schedule and recommendations are suitable for a very wide range of species. This schedule is for isolates maintained on agar slants at about 5°C with periodic transfer at 6-month intervals; a very high percentage of fungus strains will survive 12-month intervals between transfers under the conditions given. When experience shows that a given strain will not survive the conditions of this schedule, the strain must be handled separately.

- Dispense media in culture tubes (100 x 13 mm), 2.0-2.3 ml per tube.
- Plug tubes firmly with absorbent cotton; inserted portion of plug to be 1.5-2.0 cm, external portion about same length or slightly shorter.
- Autoclave to sterilize.
- Slant tubes until media solidify, top of slanted agar not to reach more than half-way up the tube.
- Label tubes indelibly with strain number or other identification.
- Using sterile transfer needle and other aseptic precautions, transfer spore inoculum from old culture and streak it on surface of new slant; avoid splitting the surface of the fresh agar. If old culture has few spores, cut into and transfer a portion of the active margin (rather than the older center) of the parent culture.
- Incubate only until sturdy, sporulating, identifiable growth has appeared. Check carefully for aberrant growth and for contaminants; reisolate if necessary.
- Poison plugs with a few drops of plug poison (SR-21) and wipe outside of culture tubes and labels with poison.

(The recommended preparation will control mites effectively; it also will prevent the growth of practically all plug and label contaminants. Do not use so much poison solution on the plugs that it runs down inside the tube. *Be certain that an identifying dye is incorporated in the solution; inform all potential handlers of stock cultures that the dye indicates the presence of poison.*)

- Allow poisoned tubes to dry overnight.
- Store in racks or trays in refrigerator at about 5°C; cover to protect from direct drafts of circulating fan. Never store field collections or working moist chambers in the same refrigerator with stock cultures.
- Transfer entire set at 6-month intervals. Handle sensitive strains by more frequent transfer, by storing at moderate temperatures, or by other special technique.

Conservation techniques that are more suitable for special groups of organisms, that require unusual equipment, or that have limited application include oil-covered slants (Buell and Weston, 1947), soil or sand cultures (Greene and Fred, 1934), drying (Hesseltine, 1947; Rhodes, 1950), freezing (Carmichael, 1956; Hwang, 1960), and freeze-drying or lyophilizing (Raper and Alexander, 1945; Fennell *et al.*, 1950; Hesseltine *et al.*, 1960). For a general review and further suggestions, see Fennell, 1960.

An additional convenient conservation method makes use of agar slants in screw-cap test tubes. Following transfer of inoculum to appropriate media, the caps must be left loose until considerable growth is made. Caps then are tightened, and the tubes are stored at 5°C. Most isolates stored in this way need be transferred only once a year; some need more frequent transfers or storage under special conditions. As with all conservation techniques, this one has drawbacks, the most serious one being the toxic effect on many fungi of the carbon dioxide built up on tightly closed screw-cap tubes. (The technique has not proved very satisfactory, for example, for mucoraceous fungi.)

Procedures for Qualitative Isolation

Selective isolation of desired fungi from infected roots, bark, stems, and other aerial plant parts, or from colonized substrates of various kinds, can be accomplished by plating small pieces of tissues directly on selective agar media. Infected plant tissues are first thoroughly washed under running tap water. Using a flamed scalpel and forceps, aseptically cut the tissue into small pieces (5 to 10 mm in length or diameter) and blot them dry on sheets of sterile filter paper. Plate 3 to 10 pieces, depending on the fungus, on the surface of solidified selective agar medium in each Petri dish. From tissues with large lesions, choice of areas from the advancing margins of the lesion will facilitate ease of isolation. After a suitable incubation (e.g., at 25°C for two days), examine the dishes both macroscopically and microscopically for the desired fungus growing out from the tissue onto the medium.

Badly contaminated tissues can be first surface-sterilized by treating them briefly (30 to 60 seconds) with disinfestants such as sodium hypochlorite (10-20% Clorox {SR-22}) or 70% ethanol, followed by three rinsings in sterile distilled water. Surface sterilization often kills also the fungus to be isolated and is not a necessary or desirable procedure especially if an effective selective medium capable of controlling contaminating fungi and bacteria is used.

For fast-growing fungi, such as *Pythium*, *Rhizoctonia*, and certain species of *Phytophthora*, qualitative isolation from infested soil can be achieved by simply plating clumps of soil, in amounts of 100-200 mg per clump, directly on the surface of solidified selective agar medium.

For various procedures involving different kinds of tissues from which to make isolations, see Tuite (1969).

Procedures for Quantitative Enumeration

Data on quantitative isolation of fungi from substrates such as infested soil or water can be obtained with selective media if the amounts of substrate used are standardized and procedures quantified. The following method is one of the most widely used for enumerating fungal populations in soil or other similar substrates.

Soil samples, collected from root zones of infected plants, are weighed out in three replicates of 10 g each (3 g minimum). The soil moisture should be taken into account when weighing the soil. For example, if the soil moisture is 12% by weight, 11.2 g are weighed to obtain 10 g of dry weight.

Prepare a proper soil dilution from each replicate with sterile distilled water (or with more viscous 0.15% water agar to prevent quick sedimentation of soil particles). The dilution factor used is dependent on the fungal population in the soil sample. When a highly selective medium is used, a low final dilution, usually in the range of 1 in 50 to 1 in 200 can be employed. Extremely low dilution (e.g., 1 in 10) may cause excessive crowding of colonies of undesired fungi and bacteria. Higher dilutions (e.g., 1 in 500) may dilute out the fungus to be isolated and will require larger numbers of plates, especially if the soils contain low populations of the desired fungus. (When a semiselective medium, selective only against bacteria and actinomycetes, is used for enumerating fungal populations in general, final dilutions of 1 in 2,000 to 1 in 10,000, depending on the soil sample, are often employed in order to obtain about 30 to 50 fungal colonies per plate.) After labeling the plates, pipette 1 ml of well-agitated soil suspension of each replicate dilution into each of a minimum of five Petri dishes.

Add stock solutions, or suspensions, of antibiotics and other selective ingredients to the flasks or bottles containing the molten agar medium at about 45-48°C, gently mix the medium, and pour 15-16 ml of the medium into each plate. Gently rotate the plate to distribute the soil suspension evenly in the medium and allow the medium to solidify. Be sure not to let the soil suspension dry out in the plates before pouring the medium.

Incubate all plates in darkness at a suitable temperature (e.g., 25°C). If room temperature is used, all plates should be shielded with heavy dark cloth or other covers, as many of the selective ingredients used in various media can either be inactivated by light (e.g., Dexon, nystatin, pimaricin) or become extremely toxic under prolonged exposure to light (e.g., rose bengal).

After a suitable incubation period (usually 2-3 days) examine the plates both macroscopically and microscopically for the desired fungus. Examine closely the suspected colonies under high power for positive identification and circle these colonies on the plate bottom. Count and record the number for each plate. Read all the plates again after one or two more days of incubation to verify any tentatively identified colonies and to spot additional colonies growing out since the first reading.

Calculate the number of recoverable propagules per gram of soil (on a dry weight basis) by multiplying the average colony number per plate (e.g., 4.5) by the dilution factor (e.g., 200) (4.5 x 200 = 900 propagules per gram of dry soil). Handle the data of all plates in each dilution replicate as a group and then average the results of the three replicates.

If precise quantitative data are not essential, other less exact and less time-consuming procedures can be used to obtain semiquantitative results.

CHOICE OF MEDIA

All fungi require several specific elements for growth and reproduction. Some common natural media, such as potato-glucose agar, malt extract-yeast extract, glucose-yeast extract, or cornmeal agar, furnish all of the nutrients essential to the growth of most fungi. For most fungi, neither vitamins nor micro elements need be added to these media. However, even these natural substances may need to be supplemented for good growth or sporulation of some fungi. Natural media have the advantage of being easily and quickly made.

On the other hand, one may wish to demonstrate the need for a specific element or effects of concentration upon sporulation. It then becomes necessary to use a medium of known composition, using pure chemicals in so far as possible. Synthetic media have the advantage in that each component and its concentration can be controlled as desired.

It is best to designate media by the source and amount per liter of the principal ingredients, carbon and nitrogen. To speak of a sucrose-potassium nitrate (10-2) medium is far more descriptive and more meaningful than to speak of a "modified Czapek's medium." Furthermore, the basal medium may be similar for a number of synthetic media. For example, such a medium may contain a carbon source (usually a sugar), a nitrogen source, KH_2PO_4 1 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, micro elements (Fe, Zn, Mn 0.2 mg each, Ca 10 mg), thiamine 100 µg, biotin 5 µg, pyridoxine 100 µg (if desired), and distilled water 1,000 ml. Agar may be added as desired at 15-20 g per liter. Under the system of designating media here suggested, a glucose-asparagine (5-1) would be composed of glucose 5 g, asparagine 1 g, plus the other components listed above.

The pH of all media should be tested and adjusted to approximately 6.0-6.5 before autoclaving at 15 pounds steam pressure (121°C) for 15 minutes. Any suggested modification in initial pH will be given in the directions for the experiments.

There are many methods of preparing media. If a large number of cultures are going to be used, it is convenient to prepare the media in one-liter lots, each in a two-liter flask. Flasks should never be more than half full when ready for autoclaving.

Agar versus Liquid Media

Agar media are the choice for most experiments in general mycology and for sporulation of fungi. In a few experiments, such as on effects of vitamins on growth, a liquid medium should be used and the dry weights of the mycelium obtained. Convenient culture vessels are 250-ml Erlenmeyer flasks, each containing 25 ml of liquid medium. Mycelium from liquid cultures may be harvested easily by pouring the entire contents of a culture flask into a fine mesh cloth spread over a funnel; slight suction is helpful. An alternative is to use filter paper disks in a Buchner funnel. The mycelium may be washed with distilled water, placed in a small weighing pan and dried overnight at about 100°C.

Another common use of liquid media is in the production of large numbers of spores or other propagules for various purposes, including bioassays. The spores can be separated easily from the mycelium by filtration through cheesecloth or glass wool. This overall method is often superior to the removal of spores from the surface of the cultures on agar media.

For primary isolation of soil fungi, wood decay fungi, and other habitat groups of fungi, agars prepared according to directions and formulae given with each habitat group may be preferred. For primary isolation of yeasts and certain other groups of fungi, shaken liquid culture is preferred.

Constituents of Media

Glucose (frequently referred to as dextrose) and other common sugars may be purchased in quantity from several supply houses. Sucrose (table sugar) may be purchased at a grocery and is of sufficient purity for most experiments.

Nitrogen sources: Yeast extract, malt extract, amino acids, ammonium compounds, and nitrate compounds may also be obtained from biological laboratory supply houses. Casamino Acids "Certified," a Difco product, is acid-hydrolyzed casein, a mixture of amino acids. It has proved to be an excellent general nitrogen source for most fungi but is vitamin-free.

The salts used in making media should be c.p. grade. It is convenient to make a standard stock solution containing Fe, Zn, and Mn, so that a given volume may be added routinely to each liter of synthetic medium. The following amounts of the given salts have been found to be satisfactory: $Fe(NO_3)_3 \cdot 9H_2O$, 724 mg; $ZnSO_4 \cdot 7H_2O$, 440 mg; $MnSO_4 \cdot 4H_2O$, 406 mg. Dissolve these salts in 600 ml of distilled water, add sufficient c.p. sulfuric acid to yield a clear solution, and add distilled water to make one liter. Use 2 ml of this solution per liter of medium. The activated charcoal used to absorb residual traces of impurities is a commercial preparation, Norit A (pharmaceutical grade). It should be used at the rate of 5 g per liter.

The vitamins may be purchased from any of several pharmaceutical supply houses. It is convenient to make up standard master solutions of each desired vitamin in 20% alcohol at the correct concentration to provide the desired final amounts in each ml of master solution. These are: Thiamine 100 µg; biotin 5 µg; pyridoxine 100 µg. These may be made up and stored in the refrigerator in 100-ml volumetric flasks.

Measurement of pH of liquid media may be done by a pH meter or, less critically but satisfactorily, by the spot plate method. On a white porcelain spot plate place a drop of Hellige (or other) wide-range indicator in one of the depressions. Add a drop of the medium. Compare the color with the standard color chart. Add solutions of 6N NaOH or 6N HCl to adjust the medium.

Sterilization of both media and glassware may be done in the autoclave. Fifteen minutes at 15 lb steam pressure (121°C) is sufficient for media in small lots. Never sterilize or melt agar media in flasks more than half full. Always allow pressure to return to zero and a few additional minutes for cooling before opening the door of the autoclave. Steam pouring from an autoclave door can cause painful burns.

Selective Agar Media

Most fungi, especially those with slow growth rates, are difficult to isolate from infected tissues, infested soils, or decaying organic matter due to the rapid and often antagonistic development of associated fungi, bacteria, and actinomycetes on the isolation media. Successful isolation of some of these fungi can be achieved by the use of selective agar media that have been specifically devised for these fungi or related fungal groups. For the isolation of fungi in general, semiselective media containing antibacterial inhibitory substances have been widely used.

Selective exclusion can usually be accomplished by any of several approaches. One is by selective inhibition, i.e., by using selective antimicrobial chemicals, primarily antibiotics, that inhibit the development of undesired fungi and most, if not all, bacteria and actinomycetes on the medium but exert little or no effect on the desired fungus to be isolated. The other approach is by selective enhancement, i.e., by providing in the medium a carbon and/or nitrogen source(s) that are assimilable only by the fungus in question and by a limited number of other microorganisms. A third approach is to use generally nontoxic chemicals or nonmetabolite substances, not as inhibitors or nutrient sources, but to create in the isolation medium a chemical environment (e.g., high acidity, alkalinity, or salinity) or physical condition (e.g., high osmotic concentration) preferentially favorable for the desired fungus to be isolated. When possible, more than one of the above approaches are used in combination to achieve the maximum efficiency. The subject has been discussed in detail by Tsao (1970).

Semiselective Media for General Use Semiselective agar media used for isolating fungi usually contain one or more antibiotics that are inhibitory to bacteria and actinomycetes, but not to most fungi. The commonly used antibiotics are streptomycin, chloramphenicol, chlortetracycline, and some others. Other inhibitory ingredients often used in fungal isolation media are such antimicrobial substances as rose bengal, oxgall, and sodium propionate, which are only slightly toxic, but not lethal, to fungi and are used primarily to restrict the spread of colonies of some fast-growing fungi (e.g., *Rhizopus, Mucor, Trichoderma*). These chemicals often stimulate sporulation as well. Although these semiselective media (M-43, M-59) have been used by some workers for isolating a given fungus or fungal group, they are generally nonspecific and are used for isolating fungi in general. For more information on other such media, see Johnson and Curl (1971).

Selective Media for Specific Fungi or Groups For isolating specific fungi, selective antifungal agents, in addition to proper antibacterial agents, must be used. The commonly used compounds are the polyene antifungal antibiotics, such as nystatin and pimaricin. Other chemicals effective for this purpose are the soil fungicide PCNB and the antibiotic cycloheximide, the latter being widely used in the isolation of many human pathogenic fungi. Three examples of these selective agar media, each for a specific plant pathogenic fungus or fungal group, are provided (M-96, M-97, M-99). For more information on other such media, see Tsao (1970).

COLOR DATA IN MYCOLOGICAL TAXONOMY

An accurate record of color is essential in the taxonomy of many fungi. In others it is highly useful but ancillary. More color data could be used on most taxa if they were available.

For some species a simple statement of the generic hue or neutral, e.g., brown, green, gray, suffices. Often such hue designation with appropriate modifier to indicate intensity or a combination of hue and saturation is adequate. However, in some groups such as most Homobasidiomycetes, certain Discomycetes and some molds, a more precise color determination, requiring reference to a color standard, is essential. Colors of colonies of *Aspergillus* and *Penicillium* growing in vitro must be carefully noted. Colors of the stipe and of the hymenium and excipulum of the receptacle are needed for Discomycetes. The agarics and boletes are groups that require not only the most color data but also the greatest precision in recording them. In these groups requirements may vary with different genera, but some or all of the following are needed for identification of any collection: (1) ranges of variation in color of surface tissues of the pileus, the lamellae or tubes, and the stipe, also the annulus and volva if present; (2) changes in these colors with age or maturity, edaphic factors, or exposure to light; (3) colors of stipe interior and pileus trama and changes in these colors upon exposure to air; (4) color changes in any of the above in response to appropriate chemical reagents and to wounding; (5) colors and color changes in exudates from cut, broken, or wounded tissues; (6) color of rhizomorphs or mycelium at the base of the stipe or in the surrounding substrate; (7) color of fresh spore prints collected on pure white paper. Such data should be recorded from fresh specimens in prime condition and under diffuse daylight (preferably north sky).

Color standards used by mycologists are based on two concepts: (1) matching specimen color to a specific color sample; (2) relating specimen color to a three-dimensional color block having arbitrary boundaries specified by the color standard. Specimen color or colors rarely match any specific color sample in a color

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standard. By applying principle (1) above, an approximation is obtained of the color nearest that of the specimen. In systems based on (2) above, comparing a specimen with the color standard gives the block of color containing the specimen color. Systems of the latter type are preferable, particularly if the outer limits of each color block are illustrated in color, for then colors away from the center of the block can be identified more accurately and quickly.

Unfortunately, because a number of different color standards are used by mycologists today, there is ambiguity in the common color terms applied. However, the Latin color terms to be found in species descriptions should follow the terminology proposed by Dade (1943). Until recently, descriptions in English often used the *Color Standards and Nomenclature* of Ridgway (1912) on which Dade's terminology is based. Ridgway has long been out of print and is now rarely available, but Rayner recently (1970) prepared a useful and inexpensive publication that provides color samples to illustrate the color terms proposed by Dade for those who continue to use them.

Among alternative systems, the *Munsell Book of Color* (Munsell, 1966) or some scheme based on it is the most common. A precise determination of color can be obtained by interpolation in this continuous three-dimensional system. Colors in the Munsell system are recorded with a numerical notation in terms of three attributes of color: hue, value, and chroma. The ten major hues in the Munsell system consist of five generic hues (Red, Yellow, Green, Blue, and Purple) and five intermediates or combinations (Yellow-Red, Green-Yellow, Blue-Green, Purple-Blue, and Red-Purple). Value is the lightness or darkness of a color in relation to a visually-equal-stepped neutral gray scale on which black has a value of 0 and white has a value of 10. Chroma is the strength or saturation of a color in relation to a neutral gray of the same value on a numerical scale starting with the neutral gray as 0.

Little used by mycologists until recently but widely used otherwise is *The ISCC-NBS Method of Designating Colors and a Dictionary of Color Names* (Kelly and Judd, 1955). In addition to much other valuable information, this book contains a set of color-name charts defining 267 three-dimensional blocks of color based on the Munsell scales of hue, value, and chroma. Rayner's publication (1970) brings the two systems together, giving for each block in the Dade system the Munsell notation and a name from the ISCC-NBS color nomenclature.

Besides Rayner's, the following inexpensive color standards are in common use:

- Kelly, K. L., ed. 1965. ISCC-NBS Color-Name Charts Illustrated with Centroid Colors. Standard Sample #2106 Supplement to Nat. Bur. Standards Circular 553. U. S. Government Printing Office, Washington, D. C. (Available through Office of Standard Reference Materials, National Bureau of Standards, Washington, D. C. 20234) $5.00
- Kornerup, A., and J. H. Wanscher. 1967. Methuen Handbook of Colour. Methuen & Co., Ltd., London. 243 p. $9.00

PART II

TAXONOMIC GROUPS

2 Protosteliida

The Protosteliida (Olive and Stoianovitch, 1966) constitute the only order of the subclass Protostelia of the Mycetozoa (Olive, 1967, 1970). There are three families (Cavosteliidae, Protosteliidae, Ceratiomyxidae) and ten genera. They are amoeboid, holozoic organisms that ingest bacteria or fungal cells (yeasts, conidia, etc.). They occur in nature in soil, on dung, and on dead vegetation. Anteriorly flagellate cells--with one long or a long and a short flagellum--are produced by the Cavosteliidae and Ceratiomyxidae. The trophic stage of protostelids varies from uninucleate amoebae to multinucleate, reticulate plasmodia. The fruiting bodies are the simplest in the Mycetozoa, each sporocarp consisting of a slender, tubular stalk topped by a single spore or a group of two to several spores. Except in *Ceratiomyxa*, sexuality has not been demonstrated.

The group appears, on one hand, to be phylogenetically connected through *Acytostelium* to the dictyostelid cellular slime molds and, on the other hand, to the myxomycetes via the simple myxomycete genus *Echinostelium*.

Isolation

The Protosteliida may be isolated from soil, mammalian dung, and such dead plant parts as stems, flowers, tree bark, and dry or fleshy fruits. These should be collected in cellophane envelopes and kept in a cool place (8°C) until plated out in the laboratory. Care should be taken that collections do not become overheated.

In the laboratory, soak the materials in sterile distilled water for 15 to 20 minutes and break into small pieces. Blot about eight pieces on absorbent paper and then arrange in clockwise order on an agar plate so that they may be easily examined under low power of a compound microscope. Soil samples may also be placed in water and poured over agar plates, after which the excess water is poured off. If a porous clay top is used on the Petri dish, the remaining water will evaporate within one day.

Two media have been found particularly effective in isolating these organisms (M-13, M-65).

The plates may be left at room temperature in the laboratory. After three or four days, examine them with a dissecting microscope or under low power of a compound microscope. If a protostelid is spotted, isolate it by touching the spores with a small piece of agar on the tip of a spear-point needle. The spores of some species are very delicate and the agar prevents excessive drying that may kill the spore during transfer. Several species have deciduous spores that may be collected by inverting the original plate over a fresh plate of agar. The spores may be transferred to one or both of the above agars, several transfers to a plate, and a small amount of a yeast or bacterium added as food associate.

It is advisable to use different kinds of food sources, separately or in combination. *Rhodotorula mucilaginosa*, *Flavobacterium* sp., *Enterobacter aerogenes (=Aerobacter aerogenes)*, *Escherichia coli*, and several unidentified yeasts and bacteria have proven acceptable thus far. Some of the naturally associated bacteria or yeasts should also be isolated, in the event the organism has a restricted diet, as it sometimes does. A supply of food microorganisms must be kept on hand for purposes of isolation and maintenance of the cultures.

If unwanted contaminating organisms are inadvertently transferred with the spores, allow the amoebae to crawl out and then transfer them successively to fresh food sources until the contaminant is removed. Single-spore cultures may be obtained by micro-manipulation. Such cultures sporulate readily.

Maintenance

Since there is no method for long-term maintenance of the Protosteliida, they must be transferred every four to six weeks. To accomplish this, a piece of agar containing amoebae or spores is streaked onto an agar slant (M-13) and a small amount of fresh yeast or bacterium added. Cotton plugs must be used in the transfer tubes, and it is important to keep the cultures free of foreign bacteria or other contaminants. The cultures may be lost if the transfer needle is not cooled sufficiently before the cells are picked up. A 14°C temperature is best for storage purposes.

Laboratory Study

Living cells are best studied in van Tieghem cell cultures. These can be prepared by attaching a glass ring to a slide with vaseline, putting a thin drop of water containing the cells on cover slip and inverting this over the ring, which has in turn been rimmed with vaseline. In a few minutes, the cells adhere to the cover slip, giving a clear, flat preparation. Alternatively, a thin block of agar can be cut out and placed over the droplet before inverting and sealing the cover slip to the glass ring. The agar exerts pressure on the cells, flattening them against the cover slip and making excellent preparations for use with the phase microscope. In such preparations, it is easy to observe cytoplasmic movement, functioning of contractile vacuoles, ingestion, food vacuoles, nuclei and cell division.

The fruiting bodies of the protostelids are best seen by making cuts through the agar culture and allowing them to develop at right angles to the cut surface. Fruiting structures usually appear within two to four days after inoculation, but the time varies according to the species. To ensure optimum fruiting, cultures should be given alternate periods of dark and light and kept at a temperature of 23°C.

3 Cellular Slime Molds

The taxonomy of the Acrasieae is unsettled, but most investigators recognize four or five families, of which the Dictyosteliaceae has been studied most intensively--indeed, to many biologists this family is synonymous with cellular slime molds. The student is referred to Alexopoulos (1962) for a general statement, to the second edition of Bonner's *The Cellular Slime Molds* (1967), and to Chapter 10 of Vanable and Clark (1968) for a more detailed account, and to K. B. Raper's 1960 article for photographic illustrations of these singular microorganisms.

The myxamoebae feed primarily by the ingestion and digestion of bacterial cells, and a wide variety of bacteria may be used for this purpose. The two organisms may be grown together if the agar substrates employed to nourish the bacteria do not yield inimical byproducts. Alternatively, the bacteria may be pregrown in broth cultures, harvested and washed by centrifugation, and streaked or spread on nonnutrient agar in a milk-white suspension. Experience indicates that the coliforms *Escherichia coli* and *Enterobacter aerogenes* are among the best food bacteria, and species of *Dictyostelium* and *Polysphondylium* are usually cultivated in association with one or the other of these in what is termed a two-membered culture. Because of its large cells, *Bacillus megaterium* is very useful for observing the ingestion and digestion of bacteria by the myxamoebae;

whereas *Serratia marcescens*, due to its intense red pigment and the inability of most myxamoebae to destroy it, provides a convenient means of labeling the cells for certain types of experiments.

Although imprecise in composition and somewhat brown, hay extract agar (Raper, K. B., 1937) (M-12) affords a satisfactory medium for the cultivation of most cellular slime molds, and when diluted to about 1/3 nutrient strength, constitutes the best isolation medium available. Such large and vigorous species as *Dictyostelium discoideum*, *D. mucoroides*, *D. purpureum* and *Polysphondylium violaceum* may be successfully grown with *Escherichia coli* or *Enterobacter aerogenes* on nutrient-rich media containing as much as 1.0% lactose and 1.0% peptone, the breakdown products of the two components tending to counterbalance each other to maintain a favorable pH. Other species, such as *Polysphondylium pallidum* and *D. minutum*, grow and fructify normally only on substrates (M-45) containing much lower levels of nutrient.

The optimal pH for these slime molds appears to be about 6.0, although a range of 5.5 to 7.0 is generally satisfactory. Phosphate buffers are commonly used.

Most cellular slime molds grow best and produce typical fructifications when incubated at or just below the temperature of the laboratory (23-25°C); a few species, e.g., *Dictyostelium polycephalum*, grow optimally at 30°C; and one exceptional strain of *Polysphondylium pallidum* grows and fruits at 37°C. Almost any species will grow and develop normally at temperatures of 20°C, or even 15°C, but at appreciably slower rates.

Light is not known either to stimulate or retard the growth of the myxamoebae of any cellular slime mold. It does, however, markedly affect the process of cell aggregation in certain species (Kahn, 1964; Konijn and Raper, 1965); and in most of the larger forms of *Dictyostelium* and *Polysphondylium* it profoundly affects the orientation, dimensions, and proportions of the sorocarps. In *D. discoideum*, characterized by a migrating pseudoplasmodium, it strongly influences the sites at which the fructifications develop. In this species, the migrating pseudoplasmodium, or slug, is very responsive to light, to slight increases in temperature, and to the ionic or solute strength of the underlying agar. For example, on a medium of low nutrient and ionic content in one-sided light at 20°C, a slug may migrate for many hours or even days and traverse relatively long distances before building an upright fructification. The same basic behavior may be observed in *D. purpureum*, *D. mucoroides*, and *Polysphondylium violaceum*, but in these species the stalk is continuous from the site of cell aggregation to the sorus, only the terminal portion approaching a vertical orientation.

Isolation

Historically, the cellular slime molds were regarded as coprophilous organisms, and some species, particularly *Dictyostelium mucoroides*, are encountered frequently on the dungs of various animals. They are now known to represent a normal component of the soil microflora and are especially abundant in the surface soil and decaying leaf litter of well established deciduous forests. For ease of isolation and the variety of forms that may be obtained, this type of source material is recommended. After removing undecomposed leaves, 10-15 g samples are collected by scraping the soil surface with the lip of a sterile glass or plastic vial. In the laboratory, one of two procedures may be followed:

- After thorough mixing, suspend about 1 g of sample in a tube containing 9 ml sterile water. Shake vigorously several times over a period of a half hour, after which streak three or four loopsful on each of three plates of dilute hay extract (1/3 strength) agar (M-12), using a loop of 4-5 mm inside diameter. Use one loopful for each streak. Incubate plates at 24-25°C and observe periodically after the second day. Cellular slime molds may be detected as aggregating pseudoplasmodia, characterized by inflowing streams of myxamoebae, or as sorocarps in various stages of development. Mature structures are easily identified by their cellular parenchyma-like stalks and their glistening, globose to citriform sori. Remove spores from selected sori with a finely pointed needle and implant near one end of a pre-inoculated streak of *Escherichia coli* or *Enterobacter aerogenes* on hay extract or dilute hay extract agar. Repeat this process when the slime mold has grown through to the other end of the streak. A second passage is usually, but not always, sufficient to rid the culture of contaminating bacteria and fungi that may have carried over with the inoculum from the primary isolation plate. Spores from sorocarps developed in the resulting two-membered culture may then be transplanted to a variety of media appropriate for the species of slime mold isolated. It is best to add bacteria from a separately maintained stock culture at each transfer.

● Clonal isolation can be made by the technique of Cavender and Raper (1965) if some estimate of the total population or the relative numbers of different species in a particular habitat is desired. To do this, suspend a sample of soil in sterile water, shake vigorously and dilute so that aliquots of 1/50 g soil in 0.5 ml suspension may be added to the surface of prepoured plates of dilute hay extract agar. At the same time, add 0.4 ml of a heavy suspension of pregrown and concentrated *E. coli* to each plate, intermix the two components thoroughly and spread evenly over the agar surface. Clones should be evident after three to four days, appearing first as cleared areas in the bacterial layer and subsequently as clusters of sorocarps, at which time positive identification to the genus and possibly the species may be made. Isolations as two-membered cultures can then be made as described above.

Of the species the student may expect to encounter in using these isolation techniques, *Dictyostelium mucoroides* is often most abundant but may be exceeded by *D. minutum*, *Polysphondylium violaceum*, or *P. pallidum* in some soils. *Dictyostelium discoideum* and *D. purpureum* occur rather infrequently, as a rule, and other species even less so.

To shift *Dictyostelium discoideum* from one bacterial associate to another is quite simple (Raper, K. B., 1951). In migrating across sterile agar, the pseudoplasmodium frees itself of bacteria, presumably as a result of continued myxamoebic phagocytosis, and any isolated sorocarp formed a few centimeters beyond the edge of the host bacterial colony constitutes a source of uncontaminated spores that may be transferred directly to an alternate bacterial associate. For the other large species it may be necessary to allow for growth through a streak of the new associate, although this is often not required if spores are taken from a sorus formed on a long stalk and hence removed several centimeters from the site of cell aggregation. For small species, such as *D. minutum*, not fewer than two passages through streaks of the alternate associate are recommended to insure a two-membered culture with that particular bacterium.

*Feeding Habits**

The myxamoebae of *Dictyostelium discoideum* and other cellular slime molds feed by the ingestion and digestion of bacterial cells. The mass expression of this feeding is strikingly evident by the clearing of bacterial streaks, as in the purification steps outlined above or by the formation of plaques in the soil plates with added *E. coli*. To observe the process at the cellular level, cultivation of the slime mold in association with *Bacillus megaterium* is recommended because of the large dimensions of these bacterial cells (Raper, K. B., 1937).

Spore germination may be followed by planting spores either in a Petri dish on a thin layer of nutrient agar or in slide mounts. In most species, germination involves a swelling of the spore content, a longitudinal splitting of the spore wall, and the emergence of the amoeboid protoplast.

Cell Aggregation

The phenomenon of cell aggregation, or pseudoplasmodium formation, is uniquely characteristic of the cellular slime molds. Whereas the aggregative phase may be observed in primary isolation plates and will certainly be seen in the steps leading to strain purification, it can be demonstrated most effectively by spreading a mixed inoculum of bacteria and slime mold spores in a broad band across the surface of a plate containing agar of low nutrient content (M-45). In cultures of this type, growth of the myxamoebae progresses uniformly throughout the inoculated area, and most of the cells approach the fruiting phase at approximately the same time. As a result, numerous and rather evenly spaced centers of aggregation appear almost simultaneously. The first-formed pseudoplasmodia are essentially symmetrical in pattern, while those that subsequently develop tend to be more irregular in outline since the contributing myxamoebae are often drawn mainly from one side. Depending in part upon the dimensions of the initial aggregation and in part upon other factors, known and unknown, all of the myxamoebae may collect into a single compact fruiting mass, or secondary centers may develop along the streams to form few to several smaller cell masses before fructification ensues.

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*This and subsequent sections emphasize *D. discoideum*, because this species is especially favorable for study, but other robust species such as *D. mucoroides*, *D. purpureum*, and *Polysphondylium violaceum* may also be used.

Progressive changes in the developing pseudoplasmodium are best observed with a low-power dissecting microscope, and the culture dish should be disturbed as little as possible during the few hours required for the completion of this process. Duplicate plates should be available for studying aggregations at higher magnifications. Myxamoebae within the streams may be studied in the living state by covering the aggregation with a clean cover slip and observing these with the compound microscope. For this, as for the vegetative feeding cells, phase contrast should be used if possible. Few, if any, food vacuoles will be seen, but the nuclei and contractile vacuoles stand out clearly, as do the anterior regions of hyaline nongranular cytoplasm. If a phase microscope is not available, pseudoplasmodia may be fixed and stained in order to show the individual myxamoebae more clearly. This can be easily accomplished by flooding the specimen with 70% ethanol containing a trace of eosin.

Cultures of *Dictyostelium discoideum* and *Escherichia coli* seeded on 0.1 L-P (M-45) agar and incubated at 23-25°C will normally show abundant aggregations after 42 to 44 hours, while *D. mucoroides* and *D. purpureum* seeded in a similar way often show developing pseudoplasmodia after 38-40 hours.

That the acrasins responsible for cell aggregation in *Dictyostelium discoideum* and *Polysphondylium violaceum* differ can be demonstrated in the following way: Inoculate plates of 0.1 L-P agar with a suspension of *Escherichia coli* in a broad band (ca. 4 x 8 cm) and allow the agar to absorb all excess water. Then add spores of one species of slime mold about 3 cm from one end and spores of the second species at an equal distance from the other end. As the myxamoebae grow outward from these points, they will reach a common frontier, and as pseudoplasmodia form in this area, their streams will be seen to overlap, the myxamoebae of each species moving toward their own centers of aggregation.

Migrating Pseudoplasmodia

Of many simple and interesting experiments that may be performed with the migrating pseudoplasmodia of *Dictyostelium discoideum*, three are described herewith:

- That the apical tip is very sensitive to light and also directs the movement of the whole body can be easily demonstrated by marking the positions and orientation of two or three migrating pseudoplasmodia, then turning the plate 90° in relation to a light source and observing the same slugs after intervals of one, two, and three hours. Cultures suitable for this and the succeeding experiment may be grown in association with *Escherichia coli* on an agar medium containing 0.5% lactose and 0.5% peptone as nutrients. A mixed inoculum of slime mold spores and bacteria is streaked near one side of the plate, which is then incubated with the opposite side of the plate nearest the light source to induce extensive migration and to obtain well isolated slugs for observation or experimentation.
- That in normal development the cells of the apical are are predestined to form the stalk can be demonstrated by cutting a migrating pseudoplasmodium into four or five equal segments with a microscalpel (fashioned by flattening a piece of platinum-iridium wire, B and S gauge 26), displacing these so that each fragment is

apart from the others, and raising the temperature slightly to induce immediate fructification. The apical fraction will then produce a relatively heavy stalk with little or no sorus and a poorly formed basal disk; cells of the other fractions remain totipotent and will produce sorocarps of normal pattern and of dimensions proportional to the cell masses. If the apical fraction is permitted to continue migration before fructification occurs, the form of the ensuing sorocarp(s) will, with increasing time, progressively approach the normal pattern (Raper, K. B., 1940).

- That the apical portion of a normal slug consists of presumptive stalk cells can be strikingly demonstrated in another way. In this case, one culture is grown in association with *Serratia marcescens* on hay extract agar (M-12) with 0.2% of added peptone to obtain reddish pseudoplasmodia. A second culture is grown with *Escherichia coli* to obtain uncolored pseudoplasmodia. Remove the tip (ca. 1/4 of whole) of one of the latter slugs with a microscalpel and replace this with a comparable tip from a red pseudoplasmodium. If the transfer is done carefully and properly, the two pseudoplasmodial fractions will merge, migration may continue, and if the sorocarp is formed within a few hours, it will show a predominantly red stalk while the sorus and basal disk are uncolored.. (See original papers by K. B. Raper {1940} and Bonner {1952, 1957} for other experiments with migrating pseudoplasmodia.)

Dictyostelium mucoroides, *D. purpureum*, and *Polysphondylium violaceum* do not produce true migrating pseudoplasmodia but initiate sorocarp formation at the sites of cell aggregation. The developing sorocarps show a light sensitivity comparable to the slugs of *D. discoideum*. Stalk formation begins almost vertically within the cell mass, which then shifts toward the light and because of its weight soon comes to rest upon the substrate. When incubated in unidirectional light, the fruiting masses of still undifferentiated cells show a very strong phototropic response, and stalks up to 6 to 8 cm in length may be formed along the agar surface before a more nearly vertical orientation is restored and the terminal stage in fructification occurs. Until this very late stage, dramatic shifts in the direction of stalk formation can be induced by rotating the culture plate with reference to the light source.

Sorocarps

Sorocarp formation in *Dictyostelium discoideum* has been described by Raper and Fennell (1952). Careful study will show that the stalk cells upon vacuolation assume the diameter required to fill the preformed sorophore sheath--if this is very thin, the cells are vertically elongate; if it is less thin, they are horizontally flattened; if it is still thicker, the cells are irregularly polygonal.

Sorocarps in different stages of development are best studied by flooding the structures with a weak iodine-70% ethanol solution, thus fixing them *in situ*. Specimens may then be removed with a fine needle, mounted in water, and a drop of chloriodide of zinc drawn under the cover slip. Alternatively, the sorocarps may be killed in iodine-alcohol (SR-15), transferred to an iodine-potassium iodide solution for a few seconds, and then mounted in a solution of zinc chloride (SR-16) or chloriodide of zinc (SR-17). With each of these reagents the sorophore sheath and the walls of stalk cells and spores show a blue to blue-purple color indicating the presence of cellulose. With the latter method, the reaction is more rapid and dramatic but the color tends to fade quickly. If a polarizing microscope is available, additional information may be gained. If the developing sorocarps are large, the cover slip should be supported with vaseline or minute glass chips to preclude serious distortion. It should be possible to discern (1) that the cellulosic sorophore sheath is expanded near its terminus, (2) that it is laid down inside the apical tip and well in advance of the level of cell vacuolation within the stalk, (3) that cells in the apical area that later form the sorophore become clearly distinct from the larger mass of spore-forming cells quite early in the developmental process, and (4) that spore differentiation begins at the upper surface of the rising sorogen and progresses downward and inward toward the sorophore.

Dictyostelium mucoroides and *D. purpureum* provide exceptional material for observing the formative, expanded terminus of the lengthening sorophore sheath, since the specimens may be fixed with iodine-alcohol (with or without added stain), covered with a cover slip, and examined directly and *in situ* with the compound microscope. In the case of very long sorocarps, one important difference may be noted: A disproportionate number of cells is expended in sorophore construction relative to the residual mass of potential spore cells.

In the genus *Polysphondylium*, as the sorophore lengthens, a posterior fraction of the ascending mass of fructifying myxamoebae lags behind, subsequently separates vertically into a number of segments, and from each of these develops a short spore-bearing branch that is anchored to the main stem much as the latter is anchored to the agar surface. The process is repeated as many as ten or more times in the tallest sorocarps before the remaining myxamoebae differentiate to form the terminal and largest sorus. Two species, *P. violaceum* and *P. pallidum*, may be readily isolated from forest soil, and in the preceding discussions primary attention has been given to the former because it grows and fruits quite well under conditions that favor the larger species of *Dictyostelium*. For purposes of laboratory study and demonstration, however, *P. pallidum* possesses certain advantages because of its more compact and generally more symmetrical sorocarps (Raper, K. B., 1960). Successful cultivation of this species requires only that substrates of considerably lower nutrient content be employed (M-12).

4 Labyrinthulales

Isolation

Labyrinthula may be isolated from various marine algae and grasses. Along the coasts of the Pacific Northwest and North Atlantic, the most reliable source is the leaves of eelgrass, *Zostera*, collected as drift. On the Southeast Atlantic coast, *Spartina* drift is a common substrate. There is little seasonal variation in the availability of *Labyrinthula*. If the collected plant material is not used for a day or two, it should be kept cold (5°C) to reduce bacterial growth.

The most successful isolation media are 1% serum-seawater agar (M-22a), modified Vishniac's medium (M-24), and trypticase-serum-seawater agar (M-22b). For isolation, cut off small pieces of the plant material, ca. 1 cm^2, and place them on the surface of the isolation medium in Petri dishes. Streptomycin and penicillin are useful to eliminate bacterial contamination but are not necessary if the plant material is blotted briefly before placing it on the plate. Diatom contamination may be controlled with the addition of 3 mg/l germanium dioxide (GeO_2) (Lewin, 1966).

Labyrinthula colonies will appear within two to seven days, moving out from the inoculum on the surface of the agar or in the agar. On the surface, the colonies superficially resemble a myxomycete plasmodium when viewed with a dissecting microscope. In the agar, the colonies appear bushy. Under higher magnification, the individual cells of the colony may be discerned.

Colonies of marine yeast, such as *Torulopsis* and *Rhodotorula*, frequently appear on the isolation plates. Several of these colonies should be subcultured on a yeast-peptone-glucose medium (YPGS) (M-25) to be used in monoxenic culture technique with *Labyrinthula* as described below.

Maintenance

Nearly all isolates of *Labyrinthula* can be maintained in monoxenic culture with a yeast. Streak the plates or slants of 1%SSA (M-22a) with a loop of yeast and inoculate directly on the yeast streak with an agar block containing *Labyrinthula* cells. The yeast cells grow very slowly on the 1%SSA and provide food for the *Labyrinthula*. Stocks may be maintained at room temperature (22-25°C) on 10 ml slants and transferred monthly. Yeast stocks are maintained on YPGS, stored at 5°C, and transferred quarterly.

Axenic culture of many isolates of *Labyrinthula* is possible on 1%SSA or TS (M-22b) with a seawater overlay. Some other isolates that cannot be axenically cultured on either of these may grow on MV (M-24) with a seawater overlay. Use sterile seawater (2 ml in a 10 ml slant) for the overlay.

Laboratory Study

The rapid gliding motility of the vegetative cells of *Labyrinthula* makes a most interesting demonstration. The colony may be observed with a dissecting microscope, but for best observations of the motility and the delicate extracellular material in which the cells glide, phase contrast microscopy is essential.

Thin Agar Cultures By pouring thin agar plates in plastic Petri dishes, good phase contrast observations may be made directly in the Petri dish, even with oil immersion lenses.

- Thin agar plates are made by pouring 1%SSA media into small plastic Petri dishes (60 x 15 mm) and then pouring the still melted media off. A thin layer of agar remains in the plate.
- To inoculate the plates with *Labyrinthula*, place a small drop of seawater on the thin agar plate. Touch the seawater drop with the surface of a block of agar with *Labyrinthula* cells on it. The cells will wash off the block onto the surface of the drop as a mass of cells. Use a three- to four-day-old transfer to inoculate these preparations.
- Place a cover slip directly over the drop on the agar.

The cells will begin to show a gliding movement within a few minutes. It is best to observe these thin agar cultures about 2 to 4 hours after inoculation, but they will last overnight if kept moist. For best phase contrast, place the Petri dish directly on the stage of the microscope.

Slide Cultures Small transfer blocks of agar may be placed in a drop of 1% serum-seawater on a slide, a cover slip placed over, and sealed with vaspar (SR-20). Within 3 hours, the cells will have spread out from the squashed agar block. On this type of preparation the fine branching and anastomosing filaments of the extracellular net material are easier to discern than on an agar surface.

Zoosporulation A few isolates of *Labyrinthula* are able to form zoospores. The following information refers to isolate L 69-18, available from David Porter, University of Georgia. This isolate forms numerous zoospores in a defined medium (GA) (M-91) modified from Goldstein and Moriber (1966) in 14-18 days from inoculation either in still Petri dish cultures or in shaken flasks. The zoospores are laterally biflagellated with a prominent eyespot and are thought to be meiospores. The cultures turn yellow to orange 1-2 days before sporulation, presumably from the formation of the eyespot material.

For additional information on the Labyrinthulales, see also Johnson and Sparrow (1961), Pokorny (1967), Perkins and Amon (1969), Porter (1969), and Stey (1969).

5 Plasmodial Slime Molds

COLLECTION, PRESERVATION, AND STORAGE

Materials required:

- Basket or other container with handle
- Various sized cardboard, plastic, or metal boxes such as pill boxes or match boxes
- Plastic bags
- Hand lens or magnifying glass
- Hunting knife
- Pocket knife
- Notebook and pencil

Remember that most slime mold fruiting bodies are small, often no larger than 1 to 2 mm in height, and sometimes inconspicuous. Then proceed to the nearest woods two or three days after a heavy, warm rain in late spring, summer, or early fall, and examine the surface of rotting logs or stumps. Look for yellow, orange, red, brown, or white fructifications in the form of tiny globes, goblets, sausage-shaped structures, or stalked forms mostly 0.5-2 mm tall. These will sometimes occur in rather large masses. *Stemonitis*, one of the most common genera of slime molds, may be recognized by its black, brown, or purplish columnar fructifications, 4-25 mm tall, always grouped in masses of five to six up to several dozen, borne on usually black, shiny stalks. Using a sharp knife, carefully remove a portion of the bark or wood bearing the fruiting bodies and place it in a box.

Strip the bark off some wet logs and examine the wood for plasmodia. Plasmodia most commonly encountered are yellow or white, and consist of a very delicate network of jelly-like threads the edge of which is in the form of a fan. Cut out a portion of the wood bearing the plasmodium and place it in a plastic bag. One may also find fruiting bodies and plasmodia on or under moist leaves.

Give each specimen a number and record in a notebook the date and locality of each collection.

Upon returning to the laboratory, spread out the specimens of fruiting bodies on a table to air dry. Do not use artificial heat if the specimens are to be used later for starting cultures. Place the wood bearing plasmodia in a glass or plastic box lined with moist filter paper or paper towelling. Place the cover on the box to make a moist chamber. Keep the box near a window (but away from direct sunlight) if fruiting is sought.

After the fruiting bodies are dry, glue them in boxes for permanent storage. Write the specimen number on or in each box. If the boxes have separate covers, be sure that the number is written on the part in which the specimen is glued as well as on the cover that will eventually bear the name of the organism. Some collectors prefer to glue the specimen on the underside of the top.

Store the boxes in an insect-proof case or drawer. Keep a container of paradichlorobenzene (PDB) in the case so as to kill any insects or mites that are present on the specimens or that may hatch out later. There is some evidence that PDB affects the germinability of spores; if one is planning to obtain cultures from the stored specimens, PDB should be avoided. Proper drying preserves slime mold structure perfectly. The air-dried specimens differ in no way from freshly collected ones.

MOIST-CHAMBER CULTURES

Slime molds may develop in moist-chamber culture from collections of bark from living or dead trees, decaying wood, animal dung, dead leaves, or debris. Such moist-chamber cultures often yield unusual species and provide material for study in places where slime mold fructifications are seldom found, such as dry areas or cities, and during the winter months when slime molds are out of season in temperate climates.

For moist-chamber cultures, collect at random thin pieces of outer bark from living trees or decaying logs and place in Petri dishes, or in glass or plastic containers with covers, lined with filter paper or paper towelling. Pour tap water or distilled water over the bark until it is thoroughly wet and let it soak overnight. Pour off all the water and place the containers on a shelf at room temperature. After about 4 or 5 days, examine periodically for slime mold plasmodia or fruiting bodies, adding small amounts of water as needed to keep the filter paper moist. Anywhere from 10% to 100% of the specimens may be expected to produce slime mold fruiting bodies, but it may be difficult to recognize them! Fruiting bodies that develop in moist chambers are often minute and many cannot be detected without a stereo-microscope. Also, many blend so well with the bark that they are difficult to detect.

SLIME MOLDS FROM THE AIR

Prepare some half strength cornmeal agar (M-8a) and pour into sterile, plastic Petri dishes about 20-25 ml per dish. Allow to solidify. Put several agar dishes in a plastic bag and drive out into the country, preferably but not necessarily near forested areas. While the car is being driven at 50 mph or more, expose dishes to the wind by holding them out the window at arm's length for about 3 minutes. Replace the covers.

Upon returning to the laboratory, incubate dishes in the dark, at room temperature (about 20-25°C). In about two weeks, small plasmodia may develop in a few of the Petri dishes and will eventually fruit. The plasmodia may be transferred to cornmeal agar and fed with pulverized sterile rolled oats as described in a subsequent section.

IDENTIFICATION

To identify a slime mold, portions of a fruiting body must be mounted on a microscope slide under a cover slip. The following points are pertinent:

- Very sharp jeweller's forceps are desirable for handling many slime mold fructifications. Very fine insect pins without heads (minuten nadeln, Ward's #14-W1080), mounted on match sticks or on glass or metal handles, are ideal for dissecting fruiting bodies under a stereo-microscope.
- Mount a relatively small amount of material and tease it apart carefully in the mounting medium (see below) under the stereo-microscope, before placing a cover slip on it.
- For temporary mounts, species without lime in such genera as *Licea*, *Cribraria*, *Dictydium*, *Stemonitis*, and *Lamproderma* are best placed in Amann's solution (SR-1), covered with a cover slip, and heated gently over a small flame. If the mount is then ringed with nail polish, it will last many weeks. More permanent mounts may be made by using CMC-9 or CMS-9AF (SR-2).

For species in which lime is present, use distilled water or a very dilute solution of detergent as mounting medium. Heat gently. If a permanent mount is desired, slowly replace the water with Karo mounting medium (SR-6) by placing a drop of the medium on one edge of the cover slip and allowing it to seep through slowly. Allow 24 to 48 hours for this process. The spores are likely to shrivel in this medium, but the lime nodes of the capillitium are well preserved.

One of the best keys for North American slime molds is Martin's (1949). An abbreviated form of this key is included in the Teacher's Guide that accompanies the three University of Iowa films on slime molds (Koevenig, 1961).

After arriving at a tentative identification by using the key, compare the specimen with the description of that species and consult an illustration, preferably one in color (Lister, 1925; Crowder, 1926; Alexopoulos and Koevenig, 1964; Martin and Alexopoulos, 1969).

LABORATORY CULTURE

Slime molds may be cultured in the laboratory either from plasmodia or sclerotia brought in from the field or from spores. Plasmodia and sclerotia are somewhat difficult to find in the field but easy to culture; spores of many species are easy to find in the field but difficult to culture.

Plasmodial Culture

The Camp method of plasmodial culture is excellent for observations of day-to-day growth and development. Take a wood block about 3 x 3 x 1/2 inches, a Petri dish cover, or some other object of similar dimensions, and wrap it in filter paper or absorbent paper of some sort. Place it in a larger glass container, such as a culture dish or finger bowl, and pour enough water into the dish so that the filter paper will act as a wick and remain constantly moist but never submerged. Place a piece of the plasmodium, or the wood bearing some plasmodium, on the moist surface of the filter paper and place four or five flakes of rolled oats (regular is preferable to quick-cooking types) in a circle around the plasmodium. Cover the culture dish with a piece of glass (Figure 2).

If a clean glass slide is placed next to the advancing edge of a plasmodium, the latter may crawl onto the slide. The slide may then be removed for observation of protoplasmic streaming under the microscope. In this case do not use a cover slip, but observe directly under low-power and high-dry objectives.

Cultures from Sclerotia

Sclerotia may be found in late fall or winter under logs or leaves. Some biological supply companies also offer them for sale. They may be used in the same way as plasmodia to start cultures.

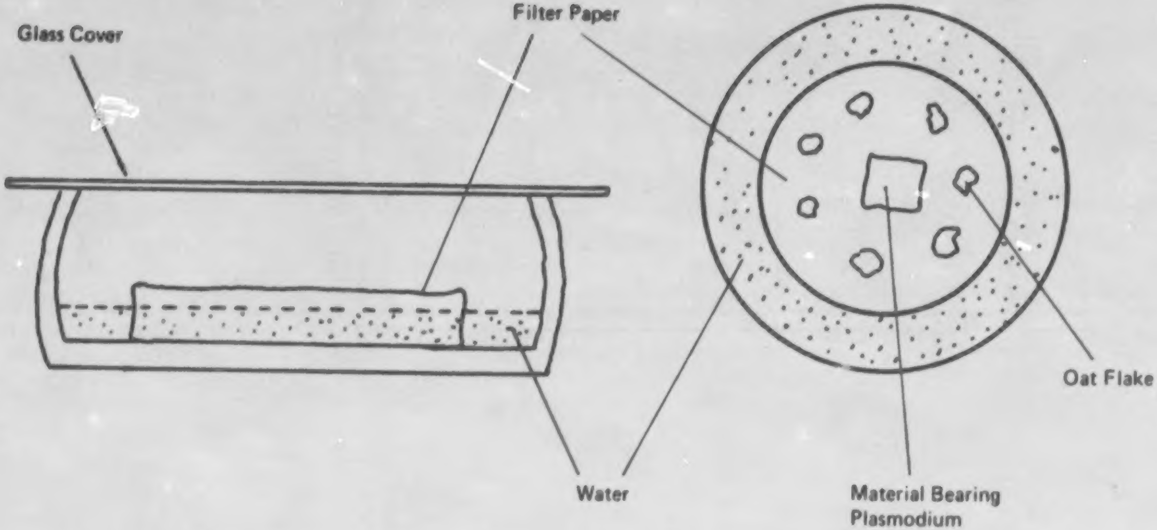
Glass Cover
Filter Paper
Water
Material Bearing Plasmodium
Oat Flake

FIGURE 2 *Diagram of culture dish as prepared for growing plasmodia of slime molds.*

Cultures from Spores

Success in culturing plasmodial slime molds from spores depends to a very large extent on finding a suitable species. The best of the commoner species for this purpose are: *Didymium nigripes, Didymium iridis, Didymium squamulosum, Physarum flavicomum, Physarum polycephalum, Physarum tenerum, Physarum gyrosum, Physarum cinereum,* and *Physarella oblonga. Fuligo septica* will also grow easily from spores but will not fruit easily in culture, whereas the other species mentioned may be carried through the entire life cycle. In tropical areas, the common *Physarum nicaraguense* may be added to the above list.

The best culture medium is half-strength cornmeal agar without glucose (M-8a). This may be purchased in one-pound bottles or prepared from the separate ingredients. Since slime mold cultures are usually contaminated with other organisms, sterile technique is not mandatory, although it is more satisfactory because fungi may otherwise spread rapidly in the cultures. After preparing the agar, pour into sterile Petri dishes in 15-20 ml quantities and allow to solidify. The deeper Petri dishes (100 x 20 mm) are better than the shallower (100 x 15 mm); for the shallower dishes, use 10-15 ml agar.

When the agar has solidified, place a sporangium on the surface, break it open by means of a steel needle or pair of forceps, and spread the spores by means of a wire loop. Pour 3-4 ml sterile water over the culture and incubate at temperature not much above 25°C. If fresh sporangia have been used, the spores should germinate in 10 to 24 hours. If older sporangia are used, germination may take a little longer; it is not advisable to try to germinate spores older than 3 or 4 years. Although it is reported in the literature that myxomycete spores retain their viability for 60 years or more in storage, the fresher the spores the better the results to be expected.

Twenty-four hours after planting the spores, examine the culture under low power by placing the open Petri dish on the stage of the microscope. Focusing on the surface of the water layer, you should see very minute swarm cells swimming rapidly, or a drop of the water can be prepared in a hanging-drop slide. Focusing on the surface of the agar, you should see spores that have cracked open and myxamoebae that have emerged. A periodic examination of the culture may reveal copulating swarm cells, but these can best be seen in a hanging-drop culture. The high-dry objective may be used to observe swarm cells through the cover slip.

When the myxamoebae population has been sufficiently built up on the agar culture, it is better to pour off any water that still remains.

About 5-6 days after spore germination, tiny plasmodia will have been formed on the agar and may be recognized as minute, rather thick, viscid "droplets" showing slow, irregular streaming under the high-dry objective. In another day or two, these droplets will have elongated and formed tiny fan-shaped anterior portions and will show rhythmical reversible streaming in the main axis of the microscopic plasmodium.

When the plasmodia are large enough to be seen without a lens, feed the culture by sprinkling sterile, pulverized oat flakes on it. Pulverized oat flakes should be dry-sterilized before use by autoclaving in dry, cotton-plugged test tubes for 1/2 hour or more.

If the cultures are contaminated with fungi, as is very likely, the plasmodia of most species can be freed from the fungus by cutting out a block of agar bearing a large portion of a plasmodium and transferring to a Petri dish containing cornmeal agar or water agar. The plasmodium should crawl off the block and migrate away from the fungus. It should then be cut out, transferred to another agar plate, and fed with sterile, pulverized oats.

Agar bearing a large, vigorous plasmodium may be cut into a number of pieces and each used to start a new culture.

Storage of Cultures

Plasmodial cultures are best stored in Petri dishes prepared as follows: Autoclave a quantity of oat flakes just as they come from the box and pour enough into a sterile Petri dish to cover the bottom completely. Pour 10 to 15 ml plain agar over the flakes. Rotate the dish slowly so as to distribute the flakes evenly and allow to solidify. Place a block of agar bearing some plasmodium that has been purified from fungal contaminants on the oat-flake agar. Seal the Petri dish with tape or rubber ring to prevent contamination. Cultures may be stored in this way for a month or more but must be transferred every 4 to 6 weeks. Do not store cultures in the refrigerator; store at room temperature.

LABORATORY STUDY

Plasmodia of some species grow vigorously but do not fruit well. Transferring a portion of a plasmodium to plain agar, to a piece of sterilized tree bark, or to filter paper will sometimes induce fruiting.

Floristic Studies with Bark Cultures

Select several uniform pieces of bark from various species of trees; all should be taken from about the same height and on the same side of each tree. Treat them as described above and observe under a stereomicroscope, beginning 24 hours after wetting the bark and continuing for 2 weeks. Compare the species produced on different pieces from the same tree, from different trees of the same species, and from different species of trees. In rare instances, mature or almost mature fruiting bodies will appear on the bark 24 to 48 hours after the bark was placed in a moist chamber.

Plasmodial Migration

With a wax pencil, mark the boundaries of a plasmodium on the underside of a dish. Measure the advance at intervals for an hour or so and calculate speed. Compare migration at different temperatures, say 15°, 20°, 25°, and 30°C. Determine whether protoplasmic streaming in the direction of plasmodial movement lasts longer than in the opposite direction.

Plasmodial Fusion

To half-strength cornmeal agar containing 0.02% neutral red, transfer portions of a vigorous plasmodium of *Physarum gyrosum*, *Didymium iridis*, or other species with a white or cream plasmodium. Prepare similar cultures on plates without dye. Store overnight in a dark place and allow the plasmodia to crawl off the blocks onto the main surface of the agar. The plasmodium on the neutral red agar should now be a brilliant red color.

Cut one strip of agar bearing a red and one bearing a white plasmodium and place them on opposite sides of a Petri dish containing plain agar or half-strength cornmeal agar (Figure 3). Allow the plasmodia to migrate toward each other. When the plasmodia come in contact, observe the zone of contact under a stereomicroscope or low power objective of a compound microscope. The plasmodia will fuse and the red protoplasm will mix with the colorless. Note how the pigment spreads.

Now place two plasmodia of different species in the same Petri dish. Choose a yellow and a white plasmodium. The two will come in intimate contact but will not fuse.

Spore Germination

Suspend spores of *Fuligo septica*, *Physarum gyrosum*, or *Physarella oblonga* in water in a test tube. Shake thoroughly. If they do not wet well, use a *very* dilute detergent solution. Suspend spores of *Stemonitis fusca* in another test tube. Pour into separate Petri dishes containing cornmeal agar. Incubate for 12 to 24 hours. Examine for presence of swarm cells. If they are present, pour off the water, place a cover slip on the agar and examine the spores under the high-dry objective. Germinated spores of the Physarales (the first group) will ordinarily have cracked open conspicuously, whereas the spores of *Stemonitis* appear to be intact; but the presence of swarm cells in the latter culture indicates that germination has taken place through a pore developed in the spore wall. This experiment may also be

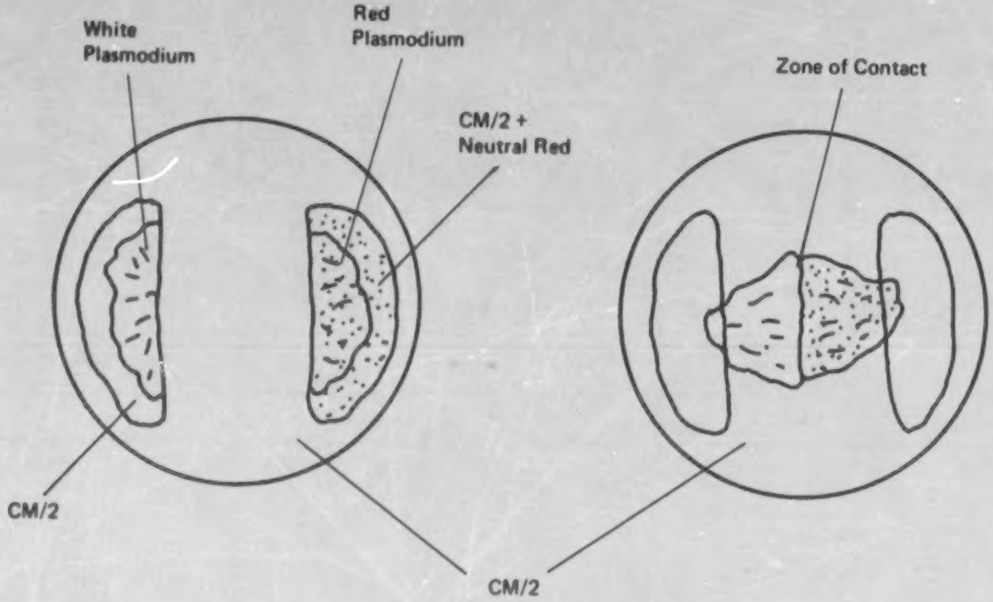
White Plasmodium
Red Plasmodium
CM/2 + Neutral Red
Zone of Contact
CM/2
CM/2

FIGURE 3 *Diagram of migrating plasmodia of slime molds. CM/2 indicates culture medium without dye. CM/2 + neutral red indicates medium with dye.*

performed using hanging drops, but unless these are properly constructed, they will dry before the spores germinate.

If incubators are available, select three temperatures (15°, 25°, and 35°C) and incubate agar plates in which spore suspensions have been poured. Examine periodically for germination. Such an experiment may be refined to include an assessment of percentage of germination by counting at intervals cracked and whole spores of Physarales within measured areas and plotting curves.

Effect of Light on Fruiting

Select four species of myxomycetes, two with white plasmodia (*Physarum cinereum* and *Didymium squamulosum*) and two with yellow plasmodia (*Physarum flavicomum* and *P. tenerum*) and culture them from spores on cornmeal agar in a light-proof cardboard box. Feed the plasmodia with pulverized sterile oat flakes periodically until they have grown considerably. For each species, transfer six uniform agar blocks bearing about the same amount of plasmodium to each of six cornmeal agar plates. This will require 24 plates. Return three plates of each species to the box and incubate the other three in the light, but near the box. Periodically examine the plates left in the light for evidence of fruiting. When they fruit, compare with the plates in the box.

Variations of this experiment may be devised by using various filters that will permit study of monochromatic light of various wavelengths as related to fruiting.

Effect of Light on Plasmodial Pigments

Start six cultures of each of the following species, by planting spores on CM: *Physarum flavicomum*, *Physarum gyrosum*, and *Didymium squamulosum*. Incubate three cultures of each species in a light-proof box and three cultures in the light. When plasmodia develop, feed all cultures with pulverized sterile oat flakes. When the plasmodia have covered the surface of the agar in the

Petri dish, compare the color of those grown in the dark with those in the light.

For additional information on plasmodial slime molds see Macbride and Martin (1934), Hagelstein (1944), Alexopoulos (1963), and Gray and Alexopoulos (1968).

6 Zoosporic Phycomycetes from Fresh Waters and Soils*

COLLECTION

The zoosporic Phycomycetes are to be found in permanent bodies of still or slowly flowing fresh waters of any size as well as in relatively small temporary ones such as ponds, puddles, vernal pools, ditches, and the like. They can also be found in great diversity in sites such as shoreline muds, soils bordering permanent bodies of water, and those farther inland; in pastures, swales, bogs, cultivated fields, barnyards. Many additional special types of habitats will suggest themselves (alkali flats, deserts, and other environmentally extreme sites), and the interested investigator would do well to spend some time in a review of all the ordinary and extraordinary kinds of habitats available to him in the vicinity of his home base.

In general it can be said that waters with healthy growths of algae and other aquatic plants will provide favorable collecting sites. "Smelly" waters, on the contrary, heavily contaminated with organic wastes from sewage or industry, are usually barren of zoosporic fungi. It should be remembered, however, that shady bogs or swamp waters and other sites low in oxygen and high in carbon dioxide provide enrichment conditions for certain interesting, fruit-inhabiting, highly fermentative members of the Blastocladiales and Leptomitales (Held *et al.*, 1969).

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*See also Emerson (1958, 1964) and Sparrow (1960), and appropriate following sections in the Guidebook.

Direct Recovery in the Field

A variety of fungi may be obtained directly from the field. Floating or submerged small animals (fish, tadpoles, insects) with a halo of fuzzy whitish threads, or twigs and fruits bearing white gummy or fuzzy pustules, should be collected in plastic bags and taken to the laboratory in a portable polystyrene refrigerator or ice bucket for necessary microscopic observation. Free water is usually not desirable during transportation, since mechanical action will often reduce fungi and substratum to a pulp. Water from the site can be carried in separate containers if necessary. It is essential that the temperature of the collected materials be maintained during transport. If the material warms up, thalli will often die and most certainly premature and undesirable zoospore discharge will be induced. Several approaches to field collection deserve emphasis here, since they yield unusual fungi.

- Collect floating and submerged twigs in the spring (April to early June). Any type is productive, except willow. Such substrata, if undecorticated, will often have patches of upright coarse hyphae on them, particularly at the lenticels. These may be Saprolegniaceae (usually *Achlya*) but could be of such rarer fungi as *Araiospora* and *Monoblepharis*. Twigs, even those not showing visible growth, should be taken to the laboratory and placed in battery jars or shallow trays of P/3 (1/3 filtered pond water and 2/3 glass-distilled water), allowing a considerable bulk of water relative to twigs, and left at 5-15°C for a week in the dark. New growth will develop, often such interesting fungi as *Monoblepharis*. Undoubtedly the low temperature acts to cut down competition. This spring collecting seems to favor certain groups of fungi not only because of prevailing low temperature but because of less competition from algae.
- Utilize spring "pollen showers" on bodies of water

from surrounding trees, especially pines. Chytrids will be the principal fungi found and these are readily seen under the microscope perched on the outside wall of the grain. Pollen is a useful and highly efficacious substratum for simple zoosporic fungi.

- Collect cast-off integuments of immature stages of various aquatic insects. Those of midges, mayflies, and certain other species often occur in great windrows in lakes at certain seasons. A curious and interesting array of chytrids is frequently found in those that have been lying in water for a few days or have been put in laboratory dishes of water for a like period (see below).

Baiting in the Field

Instead of relying upon the chance occurrence of suitable substrata in the field, "baits" of various sorts may be set out and examined at intervals. Various fleshy fruits can be used for this purpose, and the results in terms of variety of forms revealed amply repay the small effort involved. Hard green apples and small pears are particularly good, but almost any fruit with a reasonably firm texture will serve. Tomatoes, grapes, plums, rose hips, or even green bananas and sweet corn kernels, as well as twigs such as ash or birch, have been used successfully, and in the tropics a wealth of such exotic substrata as loquats, mangoes, and cashews develop a rich flora. If the fruit skin is relatively tough or waxy, pricking the surface will often hasten the establishment of aquatic fungi. To keep the bait in place and protect it from marauding fish or other animals, it is usually placed in a "trap," a small wire basket made of quarter inch galvanized wire mesh, which can be suspended in the water by nylon fish line. A shrewd collector will hide his traps well enough to avoid the attentions of inquisitive fishermen but not so well that he can't find them himself on his second round! Time for collection and examination will vary with water temperatures. Interesting genera have been found under ice in the dead of winter after 3 or 4 weeks. Summer collections, on the other hand, should be made within a week or two to avoid extensive softening of the bait. Material is transported to the laboratory in jars or plastic bags and cooled, with ice if necessary, in the summer months. Thereafter, baits should be kept in a large quantity of P/3, and continued low temperature (5-10°C) may be necessary to reduce bacterial contamination.

Baiting in the Laboratory

By far the most rewarding way of obtaining a variety of zoosporic Phycomycetes is to set up gross cultures in the laboratory. Such cultures can be regarded as a part of an aquatic site transported indoors and, potentially at least, should yield all the organisms present at the site itself. While rarely if ever realized, this is a good premise on which to initiate investigations. The following is a list of materials and habitats that have commonly yielded zoosporic fungi:

- Submerged vegetable debris
- Mud from submerged sites
- Soil samples from shore lines especially between high and low water levels
- Marshes and swales
- Pasture lands
- Cultivated soils
- Soil from sites of temporary pools (dry)
- Such special soils as beneath salt or alkali "cakes" or deserts
- Live and dead *Sphagnum* from bogs
- Soil from barnyards

Samples of soil, water, and accompanying debris are collected in small, wide-mouth jars or "whirl-pak" plastic bags and brought into the laboratory. They must be kept aerobic and cool and should be used promptly, preferably within a few hours of collection. Dried soils can, of course, be kept indefinitely.

A number of Petri dishes, crystallizing dishes, and quart jars are half filled with water (P/3), autoclaved, and cooled. Into these jars and dishes are distributed portions of the samples that have been collected including small amounts of soil and debris as well as water. Various baits are then added. In providing inoculum and bait an important consideration is the ratio of organic matter to volume of culture. Total organic matter, particularly if it is at all readily soluble, should be kept at a low level, or the cultures are likely to be quickly taken over by bacteria and protozoa and become partially anaerobic. Under these conditions few, if any, of the aquatic Phycomycetes will appear. Many different kinds of bait can be employed, and imagination should be given free rein. Some workers believe that a single type of bait should be used in a given culture; others add a variety of baits commonly used. All baits, save pollen and grubs, are usually boiled for a few minutes. Following is a list of baits that have been used with success:

- Pollen (*Pinus*, *Liquidambar*, and *Typha* are most used). The pollen is dusted over the surface of water and debris. Unlike other baits, this never seems to foul the culture, no matter how much is used.
- Split hemp seed (*Cannabis*); three halves are sufficient. Another seed of value and far easier to obtain is scarified Redtop grass (*Agrostis alba*).
- Unwaterproofed "wettable" cellophane (#300 PT Zellerbach Paper Co.) cut in 1 inch squares. Like pollen, this material does not foul cultures.
- Decolorized grass leaves; coarse algae such as *Cladophora*, *Nitella*. Two or three pieces.
- Thin pieces of vascular plant tissue such as onion skin or root tips. Two pieces, 1 inch square, or 2 short lengths of root tip.
- Small deal insects. Fruit flies recently or long dead are small enough so 2-3 will not foul the culture.
- Ant eggs and adults. Two or 3 are sufficient.
- Very small grubs (unboiled), one or two.
- Wings of insects, several.
- Integuments of aquatic insects, several.
- Shrimp exoskeleton; two to three decalcified bits, 1/2 inch square, are used (see Karling, 1945).
- Snake and lizard skin moults. Two 1/2 inch squares are used (not the large belly scales).
- Porcupine quill shavings.
- Spider webs. Roll up on tooth pick; cut off pieces.
- Baby hair (blonde preferred), defatted overnight in ether; 10-15 strands, 1 inch long.

- Human skin peelings (defatted as above); two 1/4 inch bits.
- Horn and hoof shavings, a few small pieces.

This curious array will, upon examination, show a certain logic. It includes several types of keratin, some chitinous materials, and some cellulose or cellulose derivatives.

All baits except the pollen should be examined after two days and at daily intervals thereafter. Pollen should be examined 12 hours after dusting in the gross culture and frequently thereafter. It can be readily sampled by lowering a dry clean cover slip face down onto the water surface and quickly withdrawing it. Mats of coarse filamentous fungi can be separated by playing upon them streams of water from a wash bottle; picking apart injures many strands. All manipulations are facilitated by having available several pairs of jeweler's forceps, a very long pair of forceps for retrieving material from deep water cultures, a supply of disposable 25 mm-long Pasteur pipets and a wash bottle of sterile water that delivers a thin stream. If material on slides is to be preserved, a drop of lactophenol mounting medium (SR-1) made cherry red by addition of acid fuchsin can be applied to the edge of the cover slip (round, #1) and allowed to replace the water. The mount can then be ringed with cement.

Four specific sets of baiting procedure, most likely to yield the common genera indicated, are as follows:

Saprolegnia, Achlya Autoclaved house flies (floating on the surface) or boiled hemp seeds, 2 per crystallizing dish (CD) or quart jar. Almost any fresh sample of pond water will provide *Saprolegnia* on flies within a few days. *Achlya* is equally common but is more likely to appear on seeds. Hyphae of both are characteristically coarse, stiff, and aseptate.

Aphanomyces, Pythium Autoclaved *Drosophila* flies (6-12, floating on the surface) or boiled grass blades (2 pieces about an inch long) in CD. Hyphae are much finer than those of the previous genera, and special procedures are usually required to induce zoospore discharge (see below).

Rhizophydium, Rhizophlyctis Cultures in CD or PD (standard Petri dishes) baited with one boiled grass blade, a piece of wettable cellophane, and a light dusting of conifer pollen on the surface of the water will often produce extensive growths of these common chytrids. *Rhizophydium* appears as epibiotic thalli attached to the pollen, and *Rhizophlyctis*, an orange-pigmented cellulose decomposer, forms conspicuous thalli on the cellophane. Either organism may become established on the grass.

Allomyces Boiled hemp seeds, 2 per CD or quart jar. A teaspoon of bone-dry soil, preferably agricultural, from a tropical or warm temperate area is very likely to produce thalli of this unique genus. Allow the soil to settle in the culture before the bait is added. The fungus usually appears in less than a week and is often the only Phycomycete colonizing the substratum. (Further details on *Allomyces* are provided in a special section below.)

Parasitic Fungi

Parasitic fungi are more difficult to obtain than those treated above and are discovered primarily by long hours of searching.

Algae and fungi, primarily Phycomycetes, harbor a variety of parasites that must, of course, be sought for by way of their hosts and in the habitats occupied by them. Among the green algae, the most commonly parasitized are members of the Zygnematales (including desmids), Oedogoniales, Ulotrichales, and Cladophorales. Plankton algae, including diatoms, may also bear parasites. In all algae it is difficult to be certain whether one is dealing with true parasites or simply those that have attacked moribund plants. Occasionally, but rarely, an infection of epidemic proportions will be found among members of a population of an algal species.

Such algae as scum of *Euglena* or desmids brought into the laboratory and distributed properly in Petri dishes and exposed to a north light will sometimes develop various parasites. In recent years individual parasites have been successfully multiplied and maintained by transferring infected host cells to pure liquid cultures of the same host species, such cultures being obtained from university culture collections of algae.* Enrichment of gross cultures with large numbers of individuals of an alga may be useful in stimulating parasites to develop.

Parasites of other water molds are found in gross cultures supporting growth of the host. It has been noticed that somewhat elevated temperatures are more likely to yield parasitized material.

In studying parasites of microscopic animals, as in the algae, there is no substitute for prolonged search. Gross cultures maintained under laboratory conditions frequently yield parasites of nematodes, rotifers, and similar organisms, or of their eggs. Enrichment of gross cultures by adding numbers of such animals may produce an imbalance that favors attack by parasitic fungi.

Such unusual, small, holocarpic parasites as *Olpidium*, *Myzocytium*, and *Ligniera* often occur in prodigious numbers in the root hairs and epidermal cells of various common weeds. Chickweed (*Stellaria media*), for example, from damp localities has been found to harbor such parasites at nearly all times of the year in California. Plants must be collected carefully so as not to break off the finer terminal rootlets. In the laboratory the soil is gently washed off in a shallow pan, segments of small roots are immersed in lactophenol-cotton blue (SR-1), and the material is heated gently over a Bunsen burner to hasten clearing and penetration of the stain. Thereafter, search with a dissecting microscope usually reveals root segments that are infected and can be mounted for detailed study. Both zoosporangial and resting sporangial phases are apt to occur.

Maintaining Gross Cultures

Pythium, *Saprolegnia* and *Achlya*, and other genera similar to them in general form and habitat, may be maintained for years by keeping them in quart jars about half filled with P/3 in the refrigerator. Every six months the water should be changed and one or two new halves of hemp seed added. The bottles should remain out at room temperature (24°C) for about one week, until new colonies form, and can then be returned to the refrigerator. Other more specialized or delicate genera in the Chytridiales, Monoblepharidales, or Leptomitales are very difficult to maintain in gross cultures and are best handled in axenic growths (see below).

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*The Indiana University culture collection is outstanding.

PURE CULTURE ISOLATION

Although gross cultures are instructive and often adequate for elementary classes, pure cultures provide the essential means for making the water molds perform regularly and dependably. Isolation of the larger filamentous genera is easy and is an excellent exercise for the more advanced student. Primary gross cultures can be cleaned up by serial transfer to new water (P/3) cultures on the appropriate baits. With needles and fine tweezers hyphae from young, clean cultures can then be dissected out, washed through several changes of sterile P/3, and plated on agar media. Thinly poured cornmeal agar without added glucose (Difco) is recommended for *Saprolegnia*, *Achlya*, *Dictyuchus*, *Aphanomyces*, and *Pythium*. In most instances, growth of the mycelia of these genera is rapid enough to outdistance the accompanying bacteria, and as the hyphae approach the periphery of the Petri dish, blocks of agar containing hyphal tips can readily be cut out and transferred to new cornmeal plates for a final test of purity. If particularly persistent motile bacteria or amoebae move out with the mycelium, Raper's (1937) simple van Tieghem ring technique for limiting the spread of motile contaminants on the surface of the agar can be used. *Allomyces* hyphae should be plated on YpSs/2 (M-70a). They grow considerably more slowly, but with patience and persistence, pure cultures from hyphal tips can usually be achieved.

For all zoosporic fungi the more positive and elegant method of isolation is by plating washed spores. Indeed, for many species, especially of Chytridiales, Blastocladiales, and Leptomitales, that have no unlimited mycelial growth phase, zoospores provide the only means for pure culture isolation. Various procedures have been described (Minden, 1916; Couch, 1939; Whiffen, 1941; Stanier, 1942; Emerson and Cantino, 1948; Emerson, 1950; Koch, 1957), and these and related papers should be consulted. The basic steps are as follows:

- Vigorous material with copiously developing zoosporangia must be derived either from the primary gross cultures or from cleaner, secondary, gross subcultures.
- Thalli, singly or in groups, are then dissected out, vigorously washed through several changes of water and freshly suspended in sterile P/3 to induce zoospore discharge.
- As soon as spore release begins, the free-swimming zoospores are picked up in the capillary stream of a very fine Pasteur pipette while the material is being observed with semi-darkfield lighting under a dissecting microscope. The aim is to pick up the maximum number of spores in the minimum volume of water.
- Zoospores are then washed one to several times by gently blowing them out into a drop of sterile water on a slide or Petri dish and picking them up in the pipette again. If not more than half of the spores are lost in each transfer, the operation may be considered successful!
- Following the last wash, the spores are deposited in a few drops of P/3 on a weak nutrient medium (e.g., 1/10th strength Difco cornmeal or yeast-starch {M-70a} with about 1% agar) and spread gently over the agar surface with a bent glass rod.
- Finally, if all has gone well, within 24 hours germlings can be located and cut out from between colonies of contaminants. Transfers must be made to slants with water at the base or to broth cultures, since continued proliferation will depend upon zoospore release and germling growth.

As an aid to elimination of bacteria, antibiotics are increasingly being used in primary isolation media. Half a gram each of penicillin-G-sodium and streptomycin sulfate per liter may be added to the molten agar after autoclaving and just before pouring. Concentration of zoospores by continuous centrifugation (Fuller and Poyton, 1964) or by membrane filtration (Miller, C. E., 1967) can be used to make direct samplings from aquatic habitats.

PREPARATION OF CLASS CULTURES

Precise directions for the preparation of class material from pure cultures of some 18 genera, representing the main groups of aquatic Phycomycetes, have been presented elsewhere (Emerson, 1958) and should be consulted for details. In order, however, that the essential information for the most widely used of these forms may be immediately available, selected portions of that paper have been reproduced here (by permission of the author and publisher) with minor changes where experience has led to modified procedures. The following abbreviations have been used throughout.

tt - Pyrex test tubes, 150 x 20 mm
PD - Standard Petri dishes, 100 x 15 mm
pd - Small Petri dishes, 60 x 15 mm
CD - Crystallizing dishes, 90 x 50 mm, with Petri dish lid as cover
ef - 125 ml Erlenmeyer flask
P/3 - One part filtered pond water and two parts glass-distilled water
DS1 - Dilute salt solution (M-93c-1)
YpSs - Yeast-starch (M-70a)
YpG - Yeast-glucose (M-70b)
GY5 - Yeast-glucose (M-63b)
TG - Difco powdered tryptone, 10 g; glucose, 10 g per liter
C - Difco cornmeal agar without added glucose
RS - Resting or resistant sporangia

All solid media contain 2% plain agar unless otherwise indicated. Thus YpSs/10 is one-tenth strength nutrient, but 2% agar. Liquid media, without agar, are designated as broth, and cultures are usually set up with 50 ml of medium in each 125 ml flask. All cultures are held at room temperature (ca. 22°C) in diffuse daylight unless otherwise indicated.

Since each instructor's schedule will be different, the only day designation in the following operational directions is the number of days ahead. The quantities of material are considered ample for a class of 12 students. The directions for keeping stock cultures of each organism are given first and are then followed by instructions for preparing the class material.

Chytridiales

Rozella allomycis on *Allomyces arbuscula* Stocks: On YpSs/10 slants; transfer every six weeks by blocks of agar containing infected mycelium placed in 2 ml water at base of new slant; slant tt so that agar surface is horizontal. For preparation:

- -23 (i.e. 23 days before class), start two new stocks and two pd of *Allomyces* sporophyte (see details below).
- -14, for mature RS inoculate two water cultures, in CD plus hemp seeds, with sporangia of *Allomyces*, and after 18 hours add young *Rozella* from new stocks.
- -6, for zoosporangia and developing RS start six water cultures just as above, but use young material from water culture as the *Rozella* inoculum.

Experience has shown that heavier, more uniform, and more dependable infections occur under the more natural conditions prevailing in water cultures. Hence the rather complex schedule involving transfers from stock to water culture and then to water culture again is preferable.

Rhizophydium sphaerotheca Stocks: On YpSs slants; transfer every six weeks by colonies of plants placed in 1.5 ml water at base of new slant; slant tt so that agar surface is horizontal. For preparation:

(a) for plants on agar
  - -7, start new stocks.
  - -2, put several colonies of young plants in pd of water; zoospores discharged in ca. 3 hours are then streaked on six pd of YpSs agar.

(b) for plants in water culture
  - -5, start two ef of water with pine pollen

very lightly dusted on the surface; include minimum agar with inoculum.

On pollen this is a classic monocentric chytrid growing on a classic substratum. The several discharge papillae stand out clearly, and zoospore formation and release can usually be seen. On agar the richly developed rhizoidal system is very evident.

Nowakowskiella elegans Stocks: On YpSs/4 slants; transfer a block of rhizomycelium every six weeks. The so-called rhizomycelium penetrates slowly through the agar but is so fine that it is not always readily detectable with the naked eye. Assurance of satisfactory transfer can be obtained by examination with a dissecting microscope, which reveals the fine rhizoidal strands and numerous spindle-shaped swellings. No reproductive bodies are formed under these conditions, however. For preparation:

- -35, for zoosporangia start four pd of YpSs.
- -15, inoculate six TG broth cultures, each with ca. 10 little blocks (ca. 1 mm^3) cut from YpSs plates, and place on rotary shaker at 25°C.

By -8 there will have been formed in each flask several large balls of rhizomycelium that are then washed through two changes of water, 30 min. each, and placed in water in PD. The water should be just deep enough to cover all but the tops of the balls. There must be no more than four balls per dish, and special precautions should be taken to avoid contamination. Do not disturb until ready for class. *Nowakowskiella* serves as an excellent illustration of a polycentric operculate chytrid with apophysate reproductive bodies.

Blastocladiales

Blastocladiella simplex Stocks: On YpSs slants; transfer as for *Rhizophydium*, above. For preparation:

(a) for germinating RS

- -77 and -56, prepare two stocks.
- 0 (about 7 hours before class), place RS in pd of DS1.

(b) for plants in water culture with zoosporangia and RS

- -7, start 3 CD with 1 grass blade or bits of hemp seed.
- -3, repeat.

(c) for plants on agar showing rhizoidal system and zoospore release

- -1, 30 hours before class place RS {from (a) above} in pd of DS1; after 6-7 hours streak zoospores in 12 pd of YpSs/2.

For a detailed account of this genus see special section below.

Allomyces (Euallomyces) macrogynus Stocks: On YpSs slants; transfer every six weeks by small blocks of mycelium carefully cut from the lower end of the slant and placed, right side up, at the upper end of the new culture. If precautions are taken to avoid sudden temperature changes and the consequent condensation of moisture, haploid (gametophytic) and diploid (sporophytic) stocks can be kept with little difficulty. Test tubes of new media should be made up and slanted about 4-7 days ahead so that the agar surface will dry before inoculation. Large numbers of viable reproductive structures can readily be removed from agar cultures by gently scraping the surface with a flattened transfer wire. For preparation:

(a) for germinating RS

- -84, -77, and -70, make two sporophytic stocks.
- 0, place scrapings of RS in water in pd 1-2 hours before class.

(b) for mature sporophytic thalli bearing both zoosporangia (mitosporangia) and RS (meiosporangia) in water culture

- -14, start two new gametophytic stocks.
- -4, inoculate four pd with one hemp seed with small scrapings of gametangia.
- -3, move each seed to new water in pd.

(c) for gametangia, gamete release, and syngamy

- -10, start three haploid cultures on YpSs in pd with transfers from gametophytic stocks prepared -16.
- 0, one hour before class, put scrapings of gametangia in water in pd.

(d) for germling plants showing early development and polarity

• -1, put scrapings of gametangia in water in pd 27 hours before class and streak zygotes on pd of YpSs/2 22 hours before class; repeat these operations about 8 hours later.

For a detailed account of this genus see special section below.

Saprolegniales

Saprolegnia sp. Stocks: On C slants; transfer every six weeks by blocks of mycelium cut from the lower end of the slant and placed at the upper end of the new culture. For preparation:

• -10, for proliferating zoosporangia and release of primary zoospores start two stock water cultures from agar stock by placing agar block with mycelium in CD of water with two houseflies.

• -5, using tufts of mycelium from stock water cultures as inoculum, start six CD with two flies.

Almost any *Saprolegnia* isolated from soil or muddy waters anywhere will show the characteristic sporangia and zoospores when treated in this general manner. Many isolates will also form gametangia in slightly older cultures.

Achlya sp. Stocks: On C slants; transfer as for *Saprolegnia*, above. For preparation:

• -11, for zoosporangia and zoospores inoculate C agar in two pd.

• -6, place six hemp seeds around the agar just ahead of the advancing mycelium.

• -4, remove hemp from pd and place in six CD of water.

For oogonia, oospores, and monoclinous antheridia, repeat the above steps, but start two days earlier, i.e., -13, -8, and -6. All stages in the development of the characteristic gametangia of the Saprolegniales will occur in such cultures.

Achlya ambisexualis Stocks: On C slants; transfer as for *Saprolegnia*, above. For preparation:

- -14, to demonstrate heterothallism inoculate one pd of C with the ♂ and one with the ♀.
- -7, cut blocks (ca. 5 mm^2) from the C plates and inoculate four PD of Raper's medium (soluble starch, 3 g; inositol, 1 g; peptone, 1 g; agar, 20 g; water, 1 liter) each with two blocks placed ca. 3 cm apart, two plates ♀ x ♂, one plate ♂ x ♂, and one plate ♀ x ♀.
- -5, inoculate four more PD of Raper's medium just as above.

These cultures provide a simple demonstration and basis for discussion of John Raper's (1951) classic observations on the hormone-controlled sequence of events in the sexual reproduction of *Achlya*.

Aphanomyces sp. Stocks: On C slants; transfer as for *Saprolegnia*, above. For preparation:

- -3, for zoospore discharge inoculate three ef of YpG broth (only 20 ml/flask) with five small blocks from agar stock.
- -1, 24 hours before class, divide mycelium into small tufts, wash thoroughly through three changes of water, 1 hour each, and place in three CD of water at 17°C.
- 0, about 1 hour before class, transfer to three new CD of P/3 at room temperature.

The thoroughness and timing of the washing are critical, and, in the last analysis, each instructor will probably have to work out the details of the exact schedule he uses. However, like the formation and release of zoospores in *Pythium*, zoospore discharge in *Aphanomyces* is a most exciting event and one well worth the special effort needed to produce it.

Leptomitales

Apodachlya sp. Stocks: On C slants; transfer as for *Saprolegnia*, above. For preparation:

(a) for zoosporangia and zoospores

- -8, inoculate two ef of 0.5% tryptone broth.
- -1, 18-26 hours before class, wash the mycelium through three changes of water, 10 minutes each, and

place in four CD of water at 17°C. Here again the exact timing will depend somewhat on other conditions and will have to be worked out on the spot.

(b) for gametangia and oospores

● -35, inoculate two pd of C.

● -25, use vigorous young hyphae from C pd to start six pd of GY5/2 (M-63b) agar.

Despite the small size of the gametangia, all stages in gametogenesis and oospore maturation can be studied in these cultures.

Aquatic Peronosporales

Pythium spp. Stocks: All strains are carried on C slants and transferred every six weeks as for *Saprolegnia*, above. For preparation:

(a) for sphaerosporangia and zoospores in culture (55-2)

● -10, start a stock water culture in CD with grass blades (one blade should remain in contact with the inoculum).

● -4, cut stock grass blades into pieces to inoculate six new CD of water and grass blades.

● 0, about 4 hours before class, replace the water and hold at 17°C.

(b) for nematosporangia and zoospores in culture (47-11)

● -11, start stock water culture as above.

● -4, start six new water cultures as above.

● 0, 3 to 5 hours before class, change water three times at 1/2 hour intervals and then let the grass and mycelia stand in water ca. 1 cm deep. One of mycology's real thrills is to watch a *Pythium* sporangium produce spores.

(c) for *P. aphanidermatum*

● -11, for inflated filamentous sporangia, aplerotic oospores, and smooth oogonia with paragynous antheridia start pd of C.

● -5, start six PD with water and one grass blade by using a 5 mm^2 block of inoculum (from C plate) and laying one end of the grass blade directly on it. Fine zoospore formation can usually be induced in this material by washing the mycelia just as above, but about 6 to 8 hours before class.

(d) for *P. oligandrum*

● -16, for spiny oogonia with paragynous or apandrous antheridial condition start pd of C.

● -10, start six PD with DS1 solution and two hemp seeds by using a 5 mm^2 block of inoculum from C plate and placing one hemp seed directly on the block.

Phytophthora spp. Stocks: All strains are carried on C slants and transferred every six weeks as for *Saprolegnia*, above. For preparation:

(a) for proliferating zoosporangia and direct discharge of zoospores in culture (47-11)

● -15, start pd of C.

● -8, start 12 PD with water and two hemp seeds by using a 5 mm^2 block of inoculum, from the C plate and placing one hemp seed directly on the block.

● -2, transfer hemp seed with attached myceli from the 12 PD to 12 CD of water. Typically aquatic saprophytic strains of *Phytophthora* like this, with copiously formed and conspicuously proliferating sporangia, are of common occurrence in soil-water cultures. They show the characteristic, direct discharge of spores and serve to emphasize the essentially aquatic nature of most species in this economically important genus.

(b) for oogonia and amphigynous antheridia in *Ph. drechsleri*

● -13, start pd of C of each strain.

● -9, cut 5 mm^2 blocks from the C plates, place one block of each strain in six PD and one hemp seed on each block, keep blocks well apart and add water to cover bottom of dish.

● -9, in the same manner make one control PD of "♀" x "♀" and another of "♂" x "♂".

● -5, move blocks together so that mycelia are in good contact and incubate in the dark. Male or female gametangia are not necessarily limited to one strain or the other, and gametangia not infrequently appear in the unmated control plates. However, mating of the two strains usually markedly stimulates gametangium formation.

ORGANISMS FOR SPECIAL STUDY

Because they have been used so extensively in experimental work in the past 30 years and because they can be handled so dependably in the laboratory, *Allomyces* and *Blastocladiella* offer opportunities for special study by advanced students. The following accounts have been taken almost verbatim from Lovett (1967).* For a general synopsis on developmental phenomena in these two fungi, see Cantino (1966).

Blastocladiella

Isolation from Nature Knowledge of the actual geographic distribution of *Blastocladiella* species is still very limited. They have been reported from England, France, the Dominican Republic, Mexico, New Zealand, and the United States, but the nonfilamentous restricted growth habit would tend to make them inconspicuous and easily overlooked. In most instances, species of *Blastocladiella* have been "trapped" from soil-water cultures baited with such esoteric items as boiled paspalum grass, boiled corn-seedling leaves, fly eggs, a dead fly, a hempseed radicle, and silica gel. For isolation, place 3-4 g of soil or mud from the edge of a freshwater stream, pond, or ditch in a deep crystallizing dish, mason jar, or finger bowl, with 400-500 ml of sterile 1/2 DS2 solution (M-93c-2) or P/3. As soon as the dirt has settled, float small pieces of bait on the surface and cover the container loosely. After two days at 20-25°C the bait should be examined under a dissecting microscope. When plants of *Blastocladiella* have been found, the bait can be washed a few times with sterile 1/2 DS2 (a series of 5 cm Petri dishes with 10 ml of 1/2 DS2) and transferred to a new dish with fresh bait. The growth on the second piece of bait is usually less grossly contaminated and a better source

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*"Aquatic Fungi" by James S. Lovett from *Methods in Developmental Biology* by Fred H. Wilt and Norman K. Wessells, Copyright 1967 by Thomas Y. Crowell Company, Inc. With permission of the publishers.

for isolation into pure culture. With such a nonfilamentous water mold, however, it is also wise to attempt immediate isolation.

To obtain the fungus in pure culture it is usually necessary to dissect out one or more mature plants (e.g., zoosporangia with papillae or brown resistant sporangia) under the dissecting microscope. After gently freeing the plants of as much debris as possible with fine needles, transfer with a sterile capillary pipette through several drops of sterile 1/2 DS2 (or P/3) in depression slides. After washing, leave the plants, one or more per drop, on depression slides held in a moist chamber at 20-25°C. A sterile Petri dish with several drops of sterile water and a glass-rod triangle to support the slide works well. It has the advantage that the slide can be checked under the dissecting scope without removal from the chamber. Periodic examination is then necessary to determine when discharge occurs.

As soon as possible after the zoospores have been released, transfer small droplets individually to plates of PYG agar (M-51) and spread with a sterile bent-glass rod. It is important that the plates be poured two days ahead to ensure a dry surface and prevent the spread of contaminants, but the agar should not be so dry that the water is immediately absorbed, or the delicate spores will be killed before they can germinate. If the washing was adequate, isolated plants should be present after 24 hours at 25°C. The position of those that are well separated from the nearest bacterial colonies should be marked for removal at maturity. This is done by cutting out the plant on a tiny block of agar with a fine spatula, and transferring it as before to a drop of 1/2 DS2 or P/3 to discharge. A pure culture should be obtained when the resulting zoospores are spread on fresh plates of PYG agar. Once this has been accomplished, the population can be increased by inducing several of the plants to discharge in a small volume and using this to inoculate plates or slants.

Culture Stock cultures of *B. emersonii* are conveniently maintained as resistant sporangial plants on PYG plates or slants. These are prepared by plating drops of a zoospore suspension diluted to yield small numbers of well-separated colonies (5-10 per slant or 10-50 plate). Desiccation and contamination of stocks on Petri dishes can be minimized by sealing them with large rubber bands made for this purpose or with masking tape. Slant cultures in tubes with snug metal closures keep equally well and require less storage space. Resistant sporangia can be stored this way for several months at normal room temperatures. They can also be kept on sterile filter paper strips for an indefinite period (see the procedure described for *Allomyces* below).

Working cultures of *Blastocladiella* are normally carried by daily transfer of zoospores to fresh PYG plates and incubation at 18-20°C. The time required varies as a function of cell density and temperature; crowded plants discharge earlier but reach a smaller size than those that are well separated. To transfer zoosporangial cultures, add 2-5 ml of sterile distilled water to the plate when about 5-10% of the plants have started to release spores and most of the remainder have formed papillae. Transfer two or three drops of the turbid spore suspension obtained after 15-30 minutes by pipette to a fresh plate of PYG with a few drops of sterile water. A total volume of 0.5 ml on a freshly poured 10 cm plate will ensure the retention of a film of moisture without making it overly wet. It is advisable to check the zoospore suspension each time for obvious bacterial contamination, since distinct colonies are not formed on plates with a moist surface.

When zoosporangial cultures are maintained for long periods by daily transfer, they may occasionally start to behave erratically. To avoid this apparent degeneration (the cause has not been investigated) it is a good practice to start fresh cultures from resistant sporangial stocks every month or two; spore discharge can be induced by cutting out a few mature colonies with a minimum of agar and immersing them in about 5 ml of sterile distilled water in a small Petri dish. After discharge occurs (4-8 hours at 20°C), spread several drops of the dilute zoospore suspension on each plate of PYG agar. Two or three subsequent transfers will usually bring the population density to adequate levels if the volumes used to harvest the spores are minimal. Each time new zoosporangial cultures are started it is convenient to prepare fresh resistant sporangial stocks using drops of the already dilute spore suspension.

Preparation of Zoospores The zoospore suspensions required for all synchronous cultures are harvested in basically the same way, but the procedures differ somewhat in detail depending upon the purpose for which they are to be used. Optimal yields of spores are recovered from fresh first-generation cultures of zoosporangial plants grown under conditions where a thin film of water remains at the time of discharge. The mature plants should be evenly distributed and close together on the agar surface for optimum yield and uniformity of discharge time. Zoospore formation and release is stimulated by flooding the plants at the papilla stage with small volumes of sterile distilled water. Flooding when 5-10% of the plants have started discharge will usually give the best results. Repeated washing with small volumes (3-5 ml) induces better discharge than do fewer large washings, and keeps the cell density high. The extracted PYG results in a less drastic change in the environment of the delicate spores. For inoculating cultures one such harvest from a few plates is often adequate.

Remove the spore suspension after 10-15 minutes with a sterile blow-out pipette and filter to remove any plants through a fluted porous filter paper, or a loose wad of cotton stuffed into the stem of the funnel. If the spores are not to be used at once, pack the receiving flask in crushed ice to chill the cells. In preparing sterile suspensions use an autoclaved assembly with a short-stem funnel inserted through a cotton plug into an Erlenmeyer flask of the appropriate size. Cover the funnel and upper portion of the flask with a double thickness of aluminum foil that can be raised to pipette in the spores. After filtration, a second sterile plug to fit the flask is exchanged for the funnel assembly.

The density of zoospore suspensions can be determined by counting suitably diluted aliquots with a Coulter electronic cell counter. The spores should be suspended in BSM (M-93a) at a concentration of less than 1×10^4/ml and counted using a 100μ aperture. The spores are diluted volumetrically with cold BSM, and the counts are made immediately (with frequent mixing) to avoid germination and clumping. If a cell counter is not available, the spore density can be estimated by counting drops of suspension in a hemocytometer after fixing for 15 seconds. With either method of counting it may be necessary to introduce a correction factor for viability, particularly if the spores are used in plating experiments.

When larger numbers of spores are required for fractionation, physiology, or mass germination, the procedure must be slightly modified. Now a much larger surface area of plant growth is used, say 30-40 15-cm Petri dishes. Aluminum-covered stainless steel trays as large as 21 x 16 cm can also be used for very large harvests. Sterile technique is usually unnecessary during harvesting, and the spores are concentrated and washed by low-speed centrifugation in the cold. Large yields can be obtained by flooding such cultures several times over a period of an hour or more while holding the filtered suspension on ice, and concentrating by centrifugation in 30-ml plastic tubes at 4-10°C. Bring the rotor rapidly up to speed (755 G), run for exactly two minutes, and allow to decelerate. Decant the supernatant from the very loose pellet of spores, add 2-3 ml of cold BSM (or other medium) to each tube, and resuspend the spores quickly by using a Pasteur pipette with a rubber bulb to force the liquid gently over the pellet. Wash the concentrated zoospores by pooling the resulting suspensions and repeating the procedure. After washing, take the cells up in 10-20 ml of the desired buffer, or medium, subsequent treatment depending upon what they are to be used for.

For additional details concerning the harvesting and germination of zoospores, see Lovett (1968), Soll *et al.* (1969), and Truesdell and Cantino (1971).

Synchronous Zoosporangial Cultures Conditions for growing zoosporangial plants in synchronous single-generation cultures have been provided by Goldstein and Cantino (1962). The medium contains 5.5 g Difco-Cantino PYG (M-51) per liter with 5×10^{-3}M phosphate buffer at pH 6.7. Autoclave a total volume of 1.2 liters, with a little added Antifoam A, 35 minutes at 120°C and 16 lb pressure in a 2-liter Erlenmeyer flask equipped with two aeration tubes. Inoculate the culture with 8.3×10^6 - 2.5×10^8 spores per liter and incubate with aeration (5 liters air per minute) in a water bath at 24°C. Under these conditions, generation times, defined as the stage when 5% of the plants have formed discharge papillae, of 16 hours and 17-1/2 hours have been obtained for dark- and light-grown cultures, respectively.

The method has been modified by Murphy and Lovett (1966) for the study of synchronous zoospore differentiation. Here 1 liter of the PYG-phosphate medium is sterilized and cooled in a 1-liter water-jacketed spinner flask equipped with a magnetic stirrer bar. An aeration tube, with an in-line sterile cotton filter, is inserted through one access port, and the second is plugged with cotton. Mount the entire assembly over a large magnetic stirrer and connect the jacket to a constant temperature bath at 24°C. Start the culture by inoculation with 1.34×10^7 spores prepared as described above and used as soon after harvesting as possible. Aerate the culture with a mixture of 0.5 liter tank oxygen and 1.8 liter air per minute and stir with the stirrer (Jumbo Magnetic Stirrer, Fischer Sci. Co., Chicago, Ill.) set at position 8. Grow the plants under ordinary laboratory light conditions.

The culture of synchronously developing plants is induced to form zoospores after 15-1/2 hours of growth, just before the end of the growth phase at a stage when no papillae have been formed. At this point stop aeration and mixing, allow the plants to settle for 5 minutes, and remove 75% of the medium by suction with a stick filter. Return the volume to 900 ml with 1/2 DS2 and stir the culture briefly. Repeat the exchange process, adjust the final volume with 1/2 DS2 to that at 15-1/2 hours, and resume aeration and mixing. The two changes require 20 minutes and remove 94% of the original growth medium. Under these conditions the plants remain unclumped and undergo zoospore differentiation with ca. 96% synchrony. Papillae form between the 17th and 18th hour after inoculation; visible cleavage occurs at 18-3/4 hours, and zoospore discharge 15-20 minutes later (Murphy and Lovett, 1966). By increasing the scale, this method can be used to produce large populations of zoospores.

Synchronous Resistant Sporangial Cultures For class demonstrations, small numbers of resistant sporangial plants can easily be produced by plating appropriately-diluted zoospore suspensions on plates of Difco PYG (M-51), or Difco YpSs (M-70a), agar containing 0.01 M $NaHCO_3$, or a suspension of $CaCO_3$.

The growth of large scale synchronized cultures of *Blastocladiella* resistant sporangial plants has been reported by Lovett and Cantino (1960). The medium is prepared with 5.5 g/liter of Difco-Cantino PYG broth and 8.9×10^{-2} M $NaHCO_3$. Autoclave 10 liters of medium in a 12-liter flat-bottom Florence flask equipped with a large cotton filtered aeration tube and a sealed siphon tube for removing plants. After sterilization, remove the flask from the autoclave and quickly cool it in a water bath with aeration to minimize caramelization of the medium.

Start the culture by inoculation with 1.4×10^6 zoospores per liter and incubate in a water bath at 24°C, with very vigorous aeration. The resulting plant development is well synchronized and during exponential growth the mass doubling time (2.5 hours) is about twice that of zoosporangial plants in PYG. Exponential growth ends at 26-28 hours; the decelerating growth phase between 28 and 48 hours is accompanied by septation and many physiological changes associated with the switch to irreversible development of resistant sporangia. The sporangia themselves reach maturity at 80-83 hours. The use of the siphon to remove samples permits multiple sampling over this long period without danger of contamination.

The induction of resistant sporangia by a high potassium ion concentration in continuous and batch cultures of *Blastocladiella* growing in PYG, or a peptone-glucose medium, has been reported by Griffin (1965), but the degree of synchrony in the batch cultures was not described.

Developmental Effects of Light and Dark Another species of *Blastocladiella* especially useful for some simple class demonstrations and experiments is *B. britannica* (Horenstein and Cantino, 1961). Unlike *B. emersonii*, in which alternate morphogenetic paths can be controlled by the presence or absence of exogenous bicarbonate, *B. britannica* does not respond to this salt. Instead, differentiation in this fungus is profoundly affected by radiation. In the presence of white light, zoosporangia are formed; in its absence, brown resistant sporangia. The effect of light (or, conversely, dark) is demonstrable using single generation populations grown synchronously either on PYG agar media or in liquid PYG broth, and a well-defined "point of no return" for both developmental pathways has been identified (Horenstein and Cantino, 1962). Further details are to be found in these two papers.

Production of Single-spored Zoosporangia The following procedure for producing plants that differentiate and discharge a single zoospore has been reported by Hennessy and Cantino (1972). Once a zoospore has germinated to form a tiny sporeling, the latter can, in turn, be induced to form a single zoospore again before it has had time to undergo its first nuclear division. The practical aspects of culturing such unispored plantlets are as follows:

- Prepare a spore suspension to contain between 5 x 10^5 and 5 x 10^6 spores/ml, and pass through filter paper into a flask (in an ice bath) containing 20 ml Difco PYG broth.
- Transfer the chilled spore suspension to an extra large Petri dish (or other suitable container) with glass microscope slide layered on the bottom (6 is a convenient number for the Petri dish). Spores will settle down on the slides, germinate, and stick tightly to the glass.
- Remove the slides *gently*, wash the germlings by *gently* immersing the slides in phosphate buffer (equal vols KH_2PO_4 and K_2HPO_4, 5×10^{-4} M each) for 5 minutes.
- Repeat the wash with fresh buffer.
- Lay the slides carrying the washed germlings in a new dish containing the same buffer.
- The tiny germlings are transformed into tiny "zoosporangial plantlets" that are just slightly larger than the original spores. Single papillae form on them starting about 1-1/2 to 2 hours; unispored plantlets start to release spores at about 3-1/2 to 4 hours.

This mini-cycle is a most dramatic illustration of the reduction of a fungal life cycle to its extreme lower limit.

Allomyces

Isolation from Nature Species of *Allomyces* have been isolated from many areas of the tropical and temperate regions of the world, but are most common in warmer climates. The best source for isolation is dry dirt or mud from cultivated fields or the edges of freshwater ponds, ditches, or slowly flowing streams where the soil is subject to periodic flooding. The isolation procedure outlined below is essentially that described by Emerson (1941) for *Allomyces*, but has been used over the years to isolate a variety of filamentous aquatic fungi.

To trap *Allomyces* place a few grams of soil in a deep crystallizing dish or straight-sided quart container about 2/3 full of sterile P/3 or 1/2 DS2. After the soil has settled, add one or two sterile hemp seeds for bait. These are prepared by boiling the seeds of *Cannabis sativa* (Emerson, 1958) for about 15-20 minutes to sterilize them, rupture the seed coats, and cause the white hypocotyl to protrude. They may also be cut in half with a razor blade, but this exposes an unnecessarily large surface area. To avoid excessive growth of undesirable microorganisms, it is important to keep the liquid volume-to-bait ratio large. If viable resistant sporangia of *Allomyces* are in the soil or mud, growth normally appears as a tuft of mycelium on the exposed portion of the hemp seed within two or three days at 25°C. As soon as such growth appears, it is advisable to examine it for *Allomyces* and, if present, to wash the seed (or even better a tuft of mycelium) through at least four tubes of sterile water and place it in another water culture with a new hemp seed.

Immediately after growth is established on the new seed and its identity rechecked, it should be isolated in pure culture. The simplest method is to wash the mycelial tuft as before and then aseptically break off a few hyphal filaments with a fine dissecting needle.

Using a drawn-out glass capillary transfer these through a series of drops of sterile P/3 or 1/2 DS2 in depression slides. Finally, place each hypha on a plate of YpSs agar (M-70a) poured 48 hours ahead of time to ensure a dry surface. The developing mycelia will usually grow away from any bacterial contaminants since the latter's spread is restricted by the dry surface.

A second method of isolation, particularly useful when contamination is persistent, is to isolate and plate the motile spores. To do this, break a few mature zoosporangia, or resistant sporangia, from the washed mycelium with a fine dissecting needle. Then wash these by transfer through a series of six to eight small dishes with 2-3 ml of sterile P/3 or 1/2 DS2 in each. This is most easily done with a Pasteur pipette under a dissecting microscope. Allow the sporangia to remain in the last dish, or a drop or two of liquid on a depression slide in a moist chamber, until one or more of the sporangia discharge. Spread small drops of the resulting spore suspension with a bent glass rod over the surface of two-day-old YpSs agar plates. Small plantlets (germlings) should be visible under the dissecting microscope within about 24 hours at 25°C. Any of these that are well removed from the nearest bacterial colonies can be cut out on a tiny block of agar with a small spatula and transferred to a fresh plate of medium. Hyphal tip isolations and zoospores will give rise to colonies of the sporophyte while meiospores will produce the gametophyte. A detailed description of procedures for isolating sporangia and zoospores by this method will be found in Emerson and Cantino (1948). Emerson has also successfully used antibiotics (25-100 mg/l Streptomycin sulphate, 50-200 mg/l Penicillin G) to reduce bacterial contamination during isolation (Emerson, personal communication).

Culture Stock cultures of *A. arbuscula* and *A. macrogynus* can be maintained in either of two ways, depending on whether the sporophytic or gametyphytic phase is desired. The sporophyte can be cultured by transfer of small blocks of mycelium to fresh slants of YpSs medium every six weeks, at room temperature, or three to four months at 10-12°C. Emerson (1941) has also described a procedure for storing air-dried resistant sporangia that remain viable for several years. Such stocks are easily prepared by scraping the surface of mature brown sporophytic colonies (i.e., on plates of solid YpSs) with a flat sterile blade to collect the resistant sporangia. Suspend these in distilled water and apply drops of the resulting suspension to sterile strips of filter paper. The strips can then be air dried and stored in sterile vials.

The gametophyte can be maintained only by periodic transfer of the mycelium to new slants. However, to avoid gamete discharge and consequent reversion to the sporophyte, the slants should be prepared a week in advance of transfer and the small blocks of mycelium placed right side up at, or above, the middle of the slant to avoid any traces of free moisture. Despite these precautions, brown diploid patches will sometimes appear, and care should be taken to transfer only from the yellow-orange areas of pure gametophyte. By attending to these details, cultures of the haploid phase can be carried successfully for years. If necessary, the gametophyte can be obtained, or recovered, by "germinating" mature resistant sporangia and plating the meiospores on two- or three-day-old YpSs plates. If fresh resistant sporangia are to be used, it is necessary to free them of viable diploid mycelium and zoosporangia by pipetting suspensions of sporangia onto filter paper and incubating them at 30-35°C for a few weeks, or at 50-60°C for 24 hours (Emerson, 1941). This serves not only to kill the vegetative structures but also to speed up resistant sporangium maturation and increase germination. Machlis and Ossia (1953) have provided information on the cultural factors that affect the time required for their maturation.

The diploid sporophyte can be obtained either by plating zygotes derived from the gametophyte or, less directly, via meiospores plated to yield the gametophyte and the subsequent production of zygotes. To obtain zygotes, scrape a number of the paired gametangia from the surface of haploid cultures and place in a few ml of P/3 to induce discharge. After 2-4 hours (for discharge and fusion) the zygotes in a drop or two of suspension will form sporophytic colonies after plating on YpSs agar. For routine work it is simpler to carry both growth phases in culture, but from the above it is obvious that it is much easier to obtain the sporophyte from a gametophyte culture than vice versa.

Production of "Unisexual" Hybrids The complete details for the crossing of *A. arbuscula* and *A. macrogynus*, and the isolation and characterization of the resulting hybrids, will be found in the paper of Emerson and Wilson (1954).

Production of Zoospores in Liquid Culture Carlile and Machlis (1965) have described a procedure for producing large numbers of *Allomyces* zoospores. Inoculate Erlenmeyer flasks (125 ml) containing 50 ml of liquid YpSs with zoosporangia scraped from the surface of 1-2 week old sporophyte cultures on YpSs agar. The zoospores released from the sporangia produce abundant growth after three days on a shaker at 25°C. The YpSs medium should then be decanted aseptically and replaced with an equal volume of sterile DS2 (M-93c-2).

Return the cultures to the shaker for 24 hours, by which time numerous zoosporangia will have formed. These can be induced to discharge large numbers of zoospores by transferring the mycelium to fresh DS2 in Petri dishes. Dense suspensions are produced in about two hours. Since the spores tend to settle to the bottom of the dish, it is necessary to stir them up for maximum recovery. Slight modification of the procedure should provide good material for the study of synchronized zoospore formation.

Production of Gametangia in Liquid Culture A method for inducing the semi-synchronous development of gametangia in liquid culture has been described by Turian (1963). Three media have been used that differ mainly in the time required for gametangial formation to begin: G_2Y, containing 6 g glucose and 0.5 g Difco-Yeast Extract per liter; GCY, containing 3 g glucose, 0.1 g Difco-Yeast Extract, and 3 g Difco-Casein hydrolysate per liter; and synthetic medium B (Machlis and Ossia, 1953) (M-93b) with 0.5% glucose and 0.005% yeast extract. Inoculate the cultures with small blocks of

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mycelium cut from the edge of a 6-8 day gametophyte culture on solid YpSs medium. Add from 6 to 12 blocks to 50 ml of medium in a 125-ml Erlenmeyer flask and incubate the culture on a rotary shaker at 25°C. Each block produces a colony in the form of a dense ball with the hyphal tips close together. The first stages of gametangium formation appear in G_2Y at two days, GCY at three to four days, and in the semisynthetic medium at four to five days. Only the balls formed by the inoculum blocks should be used in experiments because the small "satellite" colonies are often diploid or mixed as the result of gamete release and fusion.

Gametangial differentiation can be induced by gently washing the mycelium with sterile DS2 and resuspending it in Petri dishes of the same solution. The time required from this point on depends upon the developmental stage of the starting material. If lipid crown stage gametangia are used, the process of gamete formation in DS2 requires about two hours at 25°C. Lipid crown stage gametangia have also been harvested from cultures grown on solid synthetic medium B supplemented with a mixture of vitamins (Turian, 1963). Gram quantities (fresh weight) of male or female gametangia can be obtained by scraping the surface of several such cultures and inducing differentiation in DS2. Except for the earliest stages, this material has a great advantage over the liquid cultures because only the gametangia themselves are used. Work with the mycelial spheres has all been based on the tacit assumption that the differences observed are due to the type of gametangia produced. In addition to the problem of the relative contribution of mycelium vs. gametangia a correction factor for the presence of the agar blocks used to start the colonies is also necessary. Turian (1964) observed the production of small gametophyte plants, with precocious gametangial development, by female gametes germinated parthenogenetically in M/30 H_3BO_3 and grown in 0.1% yeast extract. It is possible this method might be developed to eliminate or minimize the difficulties associated with the block-inoculated liquid cultures.

7 Phycomycetes–Other Aspects

CHYTRIDS

Isolation of Aquatic Forms

Existing methods for establishment of axenic cultures of the rapidly growing, filamentous, eucarpic fungi, which are easily handled and readily freed of bacterial contaminants, are well known. This is less true for the nonhyphal, monocentric, aquatic Chytridiomycetes, especially most of those in the order Chytridiales, which are relatively difficult to purify. The major problem is the removing of contaminating bacteria that adhere to the small monocentric thallus. Present methods employed to purify these monocentric fungi include: isolating single zoospores in sterile water and spreading them out on a sterile agar surface; dragging, pushing, or "rolling" a single sporangium across a sterile agar surface to remove the adhering bacteria (Couch, 1939; Whiffen, 1941); streaking zoospore suspensions on suitable sterile agar media following general bacteriological techniques; and concentrating the propagules from water collections by continuous flow centrifugation (Fuller and Poyton, 1964) or by membrane filtration (C. E. Miller, 1967).

Members of the Chytridiales are quite common in water and soil. They may be found growing in or on living or dead plants and animals or their parts. Chytrids may be isolated from soil obtained from fields, gardens, lawns, etc. Water collections may be made from temporary or permanent bodies of water. Water collections should also contain small amounts of mud, dead plant materials, and rotting twigs, etc. Collections of living and moribund algae should also be brought into the laboratory.

General directions for handling chytrids after they are brought into the laboratory are to be found in the immediately preceding chapter. Once a chytrid has been recognized under the microscope, attempts can be made to isolate it. If the chytrid is growing on a pollen grain, the single grain with the attached chytrid may be transferred to a sterile Petri dish containing sterile distilled water. If the chytrid is growing on grass or other similar bait materials, the part of the bait on which it is growing may be cut from the rest of the bait and placed in another Petri dish. Additional baits should then be added to the new "enrichment" cultures so that planospores that discharge from the original chytrid sporangium will have substrates on which to grow. In this manner, a unifungal culture (one fungus only--not axenic; bacteria are likely to be present) will have been established.

The method of isolating chytrids worked out by Couch (1939) is perhaps the most precise method; it is also the most tedious. This method requires a relatively high-powered dissecting microscope (100X or more), micro-dissecting needles that have been ground down to a very fine point with the use of a whetstone (Chromel wire is inexpensive and stands flaming), and sterile 2% water agar plates. The chytrid sporangium (obtained as described above for unifungal cultures) or resting body is dissected or broken loose from the substrate and transferred to a drop of distilled water on the sterile agar surface. Using the micro-dissecting needle under the dissecting microscope, the chytrid thallus is gradually "pushed" or "rolled" over the agar surface. Bacteria contaminating the surface of the chytrid thallus adhere to the agar surface as the thallus is moved along. "Rolling" or "pushing" the thallus a minimum distance of 5 cm over the agar surface is usually necessary to free it of bacteria. The needle used in pushing the chytrid thallus must be flamed approximately every 1/2 cm of distance through which the chytrid is pushed. Flaming is best done by dipping the needle in 95% ethanol and touching the needle to the flame (this saves wear and tear on the Chromel wire needle caused by excessive, unnecessary direct flaming). Of course, the needle must be cooled each time (touched to the agar surface) before approaching the chytrid thallus again.

After the thallus has been moved at least 5 cm, it is transferred from the isolating agar surface by cutting a very small block-shaped area around it; this agar block with the chytrid thallus on it is transferred to a Petri dish or test tube containing a nutrient agar such as M_3 chytrid agar (M-92). After 12-24 hours, any bacteria still present on the isolated thallus will have reproduced enough to be seen. If bacteria are present, that particular chytrid thallus is discarded. It is generally necessary to isolate several thalli to be assured of getting at least a few that are bacteria-free. If the isolation turns out to be bacteria-free, the newly established, axenic culture can then be transferred to an agar tube and maintained in this axenic condition indefinitely (with appropriate transfers).

Synchytrium

Synchytrium is the largest genus of terrestrial chytrids and includes over 200 obligate parasites of angiosperms, mosses, and ferns, particularly of flowering plants; no species has yet been grown apart from its hosts on synthetic media. Accordingly, it cannot be maintained in culture and must be studied in the living condition or from herbarium specimens. The species may be recognized by the galls that they stimulate on their hosts. They are worldwide in distribution, but the more common ones occur on the Leguminosae.

In North America the most abundant and readily recognized species is *S. decipiens*, a short-cycled species that produces only sporangial sori and zoosporangia, which parasites the hog peanut, *Amphicarpaea*. This host grows along the ground in fairly moist and shaded woods, and *S. decipiens* appears as yellow-golden pustules on the vines and leaves. Such pustules are filled with zoosporangia that readily adhere to a dissecting needle and may be transferred to a drop of water. Fresh zoosporangia produce an abundance of posteriorly uniflagellate planospores in a short time under such conditions. However, the zoosporangia dry out quickly when kept on herbarium specimens and will not germinate. Nonetheless, such specimens will illustrate the galls as well as the size and shape of the zoosporangia.

Synchytrium macrosporum is another short-cycled species that produces only resting prosori or spores, which latter will germinate after several years as herbarium specimens if soaked in water for several weeks. It is an ubiquitous species that has been transferred experimentally from the castor bean to 1465 species in 918 genera of 176 families of flowering plants. Resting spores may be dissected out of soaked galls, mounted on moist filter paper in Petri dishes, and will germinate within a few weeks to produce planospores.

Synchytrium fulgens is a long-cycled parasite of *Oenothera*, the evening primrose that occurs under moist conditions along roadside ditches and fields, and forms reddish galls on its hosts. It produces both evanescent prosori with zoosporangia as well as dormant prosori (resting spores). Zoosporangia mounted in water produce planospores that are facultative in that they may function as zoospores or fuse to form resting prosori.

COELOMOMYCETACEAE

There is only one genus, *Coelomomyces*, the species of which are obligate parasites within the bodies of mosquito larvae, rarely adults. A few species have been found on other insects. Members of the genus have been found on all continents except South America. (See Keilin, 1921; Couch, 1945; Couch and Dodge, 1947; Couch and Umphlett, 1963.)

If the weather has been warm and the spring rains have been in progress for a week or more, living infected mosquito larvae can be found in ponds, ditches, and other bodies of water. Although the parasite may appear in any one of the four larval stages, it is more conspicuous and better for study in the fourth instar. If living parasitized larvae are not available, parasitized larvae preserved in 7% formalin and parasitized larvae mounted in lactophenol on slides can be used. The three commoner genera of mosquitoes found in the southeastern United States are *Aedes*, *Anopheles*, and *Culex*. For recognition of these genera and others in the larval and adult stages, see King *et al.*, 1960.

Using fine needles, dissect the thorax and abdomen to get some familiarity with the gut, the muscles, nerves, air tubes, etc. Some knowledge of the internal anatomy of a healthy larva is necessary for later distinguishing the mycelium and sporangia of the fungus from certain parts of the larva.

If heavily infected by *Coelomomyces*, a larva will appear yellowish or brownish, the color being caused by the mature resting sporangia, which have thick, colored walls. Mount the larva in a drop of lactophenol and with needles tear open the head, thorax, and abdomen under the binocular dissecting microscope and cover with a cover slip, avoiding air bubbles. Look for mycelium and the yellowish, oval sporangia. Put slide in a Petri dish or slide box for concentration of lactophenol. Add more lactophenol as needed over a period of several days and then seal with fingernail lacquer.

If mature fresh material of *Coelomomyces punctatus*, *C. dodgei*, or *C. lativittatus* is available, the sporangia can be brought to germination. First, tear the larva open with needles in a drop of distilled water on a slide so that the sporangia will be immersed in water. If the slide is then put in a moist chamber, such as a Petri dish with wet filter paper on the bottom, and supported on a V-shaped glass rod and left at a temperature of 70° to 80°F, the sporangia will be discharging zoospores after about 48 hours. When the zoospores are mature, their discharge may be hastened by putting the slide on which are the sporangia in an anaerobic culture dish. The same results may be obtained by covering the sporangia with a thin cover slip, number zero.

Germination of the sporangia is reproducible in the laboratory with such species as *C. psorophorae* and

C. punctatus; synchronous germination can be obtained by storing the sporangia on moist filter at 15°C for 14 days or more, then holding at 25°C for 3 days, and finally submerging in water.

PYTHIACEAE

Isolation of Phytophthora and Pythium from Soil and Roots

Collect samples of soil or rotted roots from plants suspected of having *Phytophthora* or *Pythium*. Use firm apples, free from decay by other organisms, as a selective medium. With a half-inch cork borer, bore a hole diagonally into a fruit but not through it. Pack the hole with the soil or other sample to within 1/2 inch of top and saturate with distilled water. Seal the opening with cellulose tape. Incubate at room temperature for 5-10 days.

The rot of apple caused by *Phytophthora* should be rather firm and will progress more rapidly than that caused by most other fungi. Isolate the fungus by sterilizing the surface of the apple and placing bits of rotted tissue from inside the fruit on a suitable medium (M-14, 66, 82). Check identity of fungus isolated after it sporulates.

Species of *Pythium* and *Phytophthora* can be isolated from roots by simply placing them under the agar in 2% agar plates. (See also Campbell, 1949; Middleton and Baxter, 1955; Baxter and Middleton, 1961; Klemmer and Nakano, 1962.)

Sporangia and Oospores of Phytophthora

Pure cultures of *P. erythroseptica* (both homothallic) are suitable for this exercise. Use either of two media (M-14, 82); before inoculation add a few crystals of beta-sitosterol to plates of the latter. Inoculate plates of either medium with bits of mycelium from a pure culture and incubate at 20-25°C. Place some plates in alternating light and darkness for maximum production of sporangia and others in total darkness for production of oospores. Sporangia and mature oospores should be present in 5 days.

Oogonia with paragynous antheridia are usually abundant, mostly submerged in the agar. Sporangia are more often formed on the surface. Oospore numbers will normally differ under the two conditions.

Production of Zoospores by Phytophthora

Grow pure cultures of *P. infestans* on plates or tubes of medium (M-14) at 20°C for 10 days. Collect sporangia by washing surface of mycelium with sterile distilled water and place suspension of sporangia at 10-12°C for 1-2 hrs. In most isolates of this fungus, zoospores should be released within this time. A drop or two of the sporangial suspension on a glass slide should show active release of zoospores. Isolates differ and environmental conditions are critical.

Production of Sporangia and Zoospores by Pythium

Grow species of *Pythium* (e.g., *P. vexans*) on agar medium (M-64, 66); if medium #82 is used, thiamine should be added for some species. After the mycelium has covered the agar plate (5-7 days), cut out blocks of agar, bearing mycelium, 1 cm^2 or less, and place in distilled water in Petri dishes. Under these conditions of starvation, sporangia should be formed within 2 days, especially on new mycelium growing out from agar blocks. The fungus may be maintained in this stage for several days at room temperature.

Just prior to observation, remove bits of the mycelium bearing sporangia and place in a drop of distilled water on a glass slide. Observe under low power objective at intervals of 5 min. The vesicle of protoplasm should be extruded within 10-20 min. and zoospores formed immediately. The entire process may be observed continuously under low power or high power of compound microscope.

Collection

Members of the Mucorales are found most abundantly in dung, sewage, polluted water, and soil, especially forest soil. Some mucors occur exclusively on dung of herbivorous animals, especially horse dung. The genera *Pilaira* and *Pilobolus* are good examples. *Pilaira* is often encountered in rabbit dung, and its occurrence often appears to be restricted to areas in which the same species can be found year after year. Collections should be made when the dung is as fresh as possible. It is important that a quantity be brought into the laboratory, but it should be kept in an area separate from other cultures because of mites. Care should be taken to keep the material moist, but not so wet as to promote bacterial growth.

Rat, mouse, and other rodent dung are especially good sources of the more unusual mucors, such as the parasitic species of *Syncephalis* and *Piptocephalis* that must be grown on another mucor host. The pellets of dung of these animals can be placed in Petri dishes on moistened filter paper. After fruiting occurs, isolation can be accomplished by moistening a very fine straight transfer wire in the agar (M-20) on which one wants to grow the organism. Then under a dissecting microscope one sporangium may be lightly touched with the wire and a few spores removed to establish a pure culture. However, some forms, such as *Phycomyces*, possess persistent sporangial walls. In this case it is best to remove a sporangium as it is found stuck against the rim or the lid of the Petri dish. When the sporangiophore is very short and the sporangium is not discharged, or has a persistent wall, it is well to use a pair of slender watchmaker's tweezers (flamed and cooled) and carefully pick up a sporangiophore and transfer it to a plate of weak nutrient agar (M-89). For *Pilobolus*, it is better not to attempt isolation since the medium must contain a growth factor and the pH must be maintained above 7.0. However, *Pilaira*, a closely related genus, can be isolated and grown in pure culture.

Mucor represents the basic genus of the order and can be recovered from most samples of forest soil. Usually a small amount of material taken just above the soil and at the bottom of the decaying leaf layer is best. The material should be lightly sprinkled on a weak medium, such as thin hay agar, and then the plates incubated at room temperature (never over 25°C); additional plates may be put in a refrigerator. The refrigerated plates should be incubated for two weeks or more. The plates incubated at room temperature should be examined in three or four days, and as soon as the grayish, moist sporangia are formed, they can be picked off in the manner described above for isolating mucors from dung.

Other species of *Mucor* may be isolated from stored grains, especially if the grain shows evidence of microbial growth. Most likely the species to be encountered are *Mucor racemosus* and *M. plumbeus*. From soil, one commonly isolates *Mucor hiemalis*, *M. alternans*, and *M. ramannianus*, or their close relatives.

Rhizopus, especially *R. stolonifer*, is readily obtained from fruit, especially strawberries, raspberries, and bananas. The latter usually yield *Rhizopus*, even though the fruit shows no decay. It should be remembered that most of the Mucoraceae are the first fungi that attack decaying plant material high in simple carbohydrates. Other species of *Rhizopus* may be isolated simply by sprinkling a small amount of garden soil on weak nutrient agar in Petri dishes and incubating at 37°C. Usually *R. arrhizus* can be found in this habitat.

So seldom does the genus *Phycomyces* occur in nature that a culture should be secured from a culture collection and maintained. The genus *Syzygites (Sporodinia)* may often be found in the temperate regions of the world in late summer and early fall on fleshy agarics. The agarics attacked by this genus appear soft and rotten and show a yellowish, coarse aerial mycelium growing from the mushroom. The small grayish sporangia can often also be seen. *Syzygites* can be grown readily on most media but tends to die out rather rapidly unless transferred every month. The last of the common genera in the family Mucoraceae is *Absidia*, to be found in forest soil and on such plant materials as hay, oats, wheat, other cereal grains or, especially, Brazil nuts.

The genus *Thamnidium* in the Thamnidiaceae is sometimes found on meats and on dung of carnivorous animals, but it is probably best to maintain a culture of *Thamnidium elegans* instead of attempting to get new material each year.

In the family Cunninghamellaceae, *Cunninghamella* is the most common genus and can often be found in greenhouse soil or in pine forest soil. Again, Brazil nuts usually yield members of the genus. Since all species of *Cunninghamella* grow luxuriantly on Czapek's solution agar and since practically no other mucors do, Czapek's solution agar may be used as a selective medium. In this case, sprinkle soil or other material lightly on the surface of the agar, and incubate plates at 28-31°C. If *Cunninghamella* is present, it will grow within three or four days. If plates are incubated longer, *Penicillium*, *Fusarium*, and *Aspergillus* will overrun the surface.

In the Piptocephalidaceae, the easiest genus to find is *Syncephalastrum*, which can be isolated from moldy hay and various grain samples as well as soil. It grows free of any host and bears merosporangia in large numbers. The only common species is *S. racemosum*.

In the Mortierellaceae, the genus *Mortierella* is present almost universally in forest soil, which should be sprinkled on either Czapek's solution agar or hay or water agar. Without exception, the sporangiophores will be short and small. For the best fruiting, cultures should be grown in a weak nutrient medium; Czapek's solution agar is excellent for this even though growth will be very thin. Often the simplest way to study this delicate fungus is to add a drop of alcohol to the surface of the agar, place a cover slip over the area, and study the fungus in place. A number of species fruit very well in mixed culture with other fungi and bacteria but become sterile in pure culture.

Of the Choanephoraceae, only one species is found in temperate regions, in and on the faded blossoms of cucurbits, especially pumpkin and squash. One should examine the faded wet blossoms after a day or so of damp weather, or when heavy dews have occurred late in the summer, after the cucurbit plants have bloomed for some time. One can often see a black whiskery mold on the outside as well as the inside of the soggy flowers. The temperate climate species is *Choanephora cucurbitarum*. In the tropics, *Hibiscus* and cucurbits almost always yield members of this genus and of *Blakeslea*. In humid weather, cotton blossoms are covered with members of this family.

Species of Kickxellaceae grow more slowly and are smaller than members of the other families. They can be found on rodent dung, but it is best to use a culture of *Coemansia* for class work. In certain areas, such as the desert, the family is better represented than elsewhere. In general, however, one cannot expect to find members of the family regularly when wanted.

Preservation

All the common Mucorales may be kept in a refrigerator from six to eight months in pure culture on potato glucose agar (M-20) slants. The exceptions to this are the genera *Choanephora* and *Blakeslea*, which should be allowed to stand at room temperature because they rapidly die at 4-5°C. Members of the genus *Pilobolus* are best seen directly on the dung.

After the cultures have grown at their optimum temperature for 10 days to 2 weeks, the cotton plugs should be poisoned with an agent (SR-21) to prevent entry of mites or growth of other fungi. A satisfactory preparation for poisoning plugs is described on page 66 of Raper and Fennell, 1965. Screwcap tubes are never very satisfactory for mucors. The only genus that is difficult to maintain in culture is *Syzygites*, which often dies out, but material can usually be reisolated in the late summer and fall.

When cultures are to be restarted, a sterile transfer needle or small loop is moistened in sterile agar, the cottony surface of the stock culture is gently touched, and the spores are transferred to new media. Growth usually can be seen with the naked eye the next day. For a large number of cultures, lyophilization is the preferred method of preservation. This method is described by Raper and Alexander (1945).

Presentation

All material should be presented for class work in Petri dish cultures at an age of 5 to 10 days. One of the best media for obtaining luxuriant growth is the same medium used for preserving cultures, namely, potato glucose agar (M-20) For diagnostic determination of species, a synthetic medium should be used. The most common one is asparagine glucose agar (M-81). In addition, it is necessary to use a weak nutrient medium and to observe the substrate mycelium, branchings of the sporangiophores, and presence or absence of rhizoids and stolons. For many mucors, especially species of *Mucor*, *Rhizopus*, *Absidia*, and *Mortierella*, Czapek's solution agar is used (M-89). This medium gives extremely poor growth, but fruiting does occur in many common species, and the fruiting stalks are clearly seen when viewed under a dissecting microscope. As an alternate medium, a very weak decoction of hay in agar works very well. Cultures should be examined before the colonies cover the agar in Petri dishes, to ensure that all stages of development may be observed.

One should make observations on undisturbed cultures and also prepare microscopic mounts by teasing out a small amount of the aerial portion of the colony. With the larger mucors, it is not necessary, in fact it is undesirable, to take any of the agar when the mount is made. Mounts should be made from the richer medium, which will ensure the presence of sporangia, sporangiospores, conidia, and columellae. Since the mucors are very delicate, mounting fluids are not used. Routinely a drop of 70% ethanol should be added to the material on the slide, followed by a drop of water. If the slide is to be kept for any length of time, a drop of cotton blue in lactophenol may be added at either side of the cover slip, and as the water in the mount evaporates, the material gradually takes up the lactophenol. Such mounts may later be sealed for semipermanent specimens.

Sporulation

Morphology and Production of Asexual Spores and Sporangia Using such genera as *Mortierella*, *Mucor*, *Rhizopus*, *Phycomyces*, *Thamnidium*, *Cunninghamella*, *Syncephalastrum*, *Mycotypha*, inoculate with spores or bits of mycelium near edge of agar plates and incubate near 25°C or at room temperature. Allow three days for *Mortierella*, *Mucor*, *Rhizopus*, *Cunninghamella*, and *Syncephalastrum* and five days for *Phycomyces*, *Thamnidium*, and *Mycotypha* before use in class. The tall sporangiophores of *Phycomyces blakesleeanus* can best be demonstrated by using 500 ml Erlenmeyer flasks as culture vessels and incubating in total darkness at 20-25°C.

Morphology and Effects of Environmental Factors Production of spore forms in *Choanephora cucurbitarum* is affected by environment. The suggested agar medium is M-64 or M-82. Use asexual spores or bits of young mycelium as inoculum. For the production of conidial heads (sporangioles) select Petri dishes with loose-fitting lids, or raise lids with small metal strips for better aeration. Incubate in alternate light and darkness at 20-25°C. Place other plates in continuous light and others in continuous total darkness for 3-4 days. A comparison of relative number of conidial heads will show light and dark requirements.

For the formation of sporangia place inoculated plates under high humidity in a moist chamber at 25-30°C for 3-4 days. Under favorable conditions both types of asexual spores may be formed simultaneously. (See also Barnett and Lilly, 1950, 1955.)

Mating Experiments

Cultures to be mated should be placed on potato glucose agar. Although a weak medium does allow better observation of zygospores, in many instances only the richer substrate gives normal and abundant material, with the exception of certain homothallic mucors that form zygospores on Czapek's solution agar. It is best to place the + and - strains in Petri dishes as blocks of mycelium cut from actively growing + and - cultures. If the species being used grows slowly, the blocks should be placed close together; for more rapidly growing species, the blocks should be placed farther apart. If the cultures grow at about the same rate, the blocks should be placed at one side of the plate, a position that ensures a line of zygospores ranging from mature ones to the very earliest stages at the line of union.

Observations should be made of the zygospores in place and in mounts as prepared above. For routine class work, it is advisable to depend on only a few sets of mating cultures, which need to be carried in stock. *Mucor hiemalis*, *Phycomyces blakesleeanus*, and *Rhizopus stolonifer* are probably sufficient. The first species gives a large number of zygospores regularly; *Mucor mucedo* is a much more difficult species from which to get good material. *Rhizopus* develops zygospores diffusely through the culture and is the example often illustrated in books. *Phycomyces* shows tong-like outgrowths from hyphae, the suspensors. If zygospores of other genera are desired, it is simpler to use the homothallic species of *Mucor*, *Zygorhynchus*, *Rhizopus*, *Absidia*, *Piptocephalis*, *Cunninghamella*, or *Mycotypha*, to name a few. One can illustrate the homothallic condition by using a species of *Zygorhynchus* or *Absidia spinosa*, for in these forms it is very easy to observe that both gametangia originate from the same filament.

Homothallism and Sporulation in Syzygites Use pure culture of *S. megalocarpus (Sporodinia grandis)* isolated from decaying mushrooms, which can often be found after prolonged rains in summer. A special medium is required for abundant production of zygospores (M-64) with 80 g glucose and 2 g yeast extract. Inoculate with spores or bits of young mycelium at the edge of agar plate and incubate near 25°C in darkness, or alternating light and darkness. Zygospores in all stages of development can be expected after 3-4 days, and are large enough that they can be studied easily under the stereoscope. For best production of sporangia, use medium M-64 and incubate in alternate light and darkness.

Because some isolates of this species produce few or no zygospores in culture, it would be best to test the isolate before using it in class. This species dies out quickly in the laboratory and must be transferred frequently.

Heterothallism and Sporulation in Phycomyces Use pure cultures of + and - isolates of *P. blakesleeanus*. Agar medium M-64 or M-66 is satisfactory. Because the old mycelium and sporangiophores of this fungus are so tough and difficult to cut, and spores germinate poorly without special treatment, it is best to inoculate a few plates about three or four days before their use as inoculum. Small pieces of agar bearing young hyphae can then be cut easily and used to inoculate new plates as desired. Place inoculum of + and - sexes on opposite sides of the plate about 2 inches apart, and incubate for 5-6 days near 20°C. Zygospores may fail to form if the temperature is too high, but should be present in all stages of development in a line where the two mycelia meet near the center of the plate. The bright yellow color is due mainly to beta-carotene. Streaming of protoplasm can often be observed in young cultures.

Mucor hiemalis or *Choanephora cucurbitarum* may be substituted for *Phycomyces blakesleeanus* but are less desirable as demonstration material.

8 Plasmodiophora

In the United States, *Plasmodiophora brassicae* is found in areas where cabbage or other crucifers have been grown commercially for many years. Populations of the organism will have increased in certain fields and can be reliably found in the same spots year after year. Local extension agents, growers, and field men associated with marketing and processing of cabbage, cauliflower, broccoli, or Brussels sprouts will know where these spots are in an area. The organism can also usually be found in weeds in infected areas. Clubroot usually appears in a field as spots up to several hundred feet in diameter, often in depressions or low areas. Plants appear stunted and wilt down in the heat of the day; because they regain their turgidity at night, the disease may not be particularly noticeable early in the season before severe stunting occurs. The most prevalent race of *P. brassicae* in the United States is Race 6, which attacks all standard varieties of cabbage and cauliflower in the United States (Williams and Walker, 1963).

Collection

Clubbed roots are initially whitish to cream, but within a few weeks they begin to darken and rot in the soil. It is best to collect the white clubs rather than the rotting galls, because the young clubs will be freer of bacterial and fungus rot organisms. Clubs on large cabbage plants will be about the size of a man's fist and fingers, those on cruciferous weeds will be considerably smaller. Clubbed plants should be loosened and dug from the soil with a fork rather than pulled, since the clubs are more brittle than roots and may break off while still in the soil.

Preparation and Storage

Fresh clubs should be scrubbed in running water, placed in polyethylene bags, and frozen in a deepfreeze. Under these conditions, spores in the clubs will remain viable for at least 2-3 years and provide a source of spores for inoculum.

Examination

When clubroot is to be studied, spores should be extracted from clubbed tissue and mixed with soil for infection and club development. Extract spores from the clubs, thawed or frozen, by macerating about 100 g of frozen clubs in 200-400 ml of water at high speed in a homogenizer or Waring blender. The macerate should be filtered through 4-8 layers of cheesecloth and the debris discarded. Centrifuge the filtrate for 7 min at about 2000-5000 RPM to pellet the spores; after discarding the supernatant liquid, resuspend the spores in water and pellet by centrifugation. Spores should be washed repeatedly in this manner 3-5 times, depending on the purity of the spore preparation required. Repeated washing removes bacteria and cell debris. If a centrifuge is not available, spores may be purified by allowing them to settle in a large graduated cylinder for 1/2 hr, decanting the water layer and resuspending the spores again in a large volume of water. In mass, the spores appear olive or gray and have the consistency of clay.

After washing, purified spores can be suspended in water and stored at 4°C in a refrigerator and will remain viable up to a year or more. After final suspension in water, the spores should be counted with a bright line haemocytometer and the concentration per ml recorded on a stock bottle. In counting the spores, care should be taken not to mistake small starch grains, which may also be in the pellet, for spores. The latter are uniformly spherical, whereas starch grains are slightly oval and exhibit strong birefringence under polarized light. A little iodine-potassium iodide in the counting solution will also turn the starch granules blue, distinguishing them from spores of a similar size.

Establishing Infections

Soil type and soil moisture relations are critical in establishing clubroot infections. The ideal soil type is a mixture of one part peat moss and one part black organic muck soil. Soil components should be passed through a quarter-inch mesh screen. If muck soil is not available, a rich compost will suffice. The soil mix should be steamed for 2 hr, prior to adding spores, to remove other soilborne pathogens and damping-off organisms. Mix spores with the soil very thoroughly at the rate of 10^8 spores per cc of soil, a level of inoculum that should insure heavy uniform infection of seedlings. Then transplant week-old seedlings of cabbage, cauliflower, candytuft, or other crucifers into pots containing the soil-inoculum mixture; five seedlings per 2 1/2-inch pot works well. The pots should then be sunken up to their rims in pans or greenhouse bench filled with peat moss, and the whole kept moist at all times to assure a uniform soil moisture level. Plants should be fertilized once or twice a week with a complete nutrient solution such as Hoagland's solution and grown at 70-75°F. As with many obligately parasitic fungi, when the host plant is growing best, the parasite will also thrive. About 16-18 days after transplanting, the first signs of clubbing will appear on the upper main root and lower hypocotyl. From 14-28 days the clubs will expand rapidly as the parasite gorws within the cells. At about 28 days the parasite begins to sporulate within the host cells, and the rate of growth of the clubbed root declines. By 40 days after transplanting, most clubs contain millions of mature resting sporangia that may be used as further inoculum. The *P. brassicae* soil mixture can be saved, stored in a cold room for up to several years and reused without sterilization for 2-3 successive tests.

Root Hair Infection

Root hair stages of clubroot infection can be viewed in the living condition or after staining young roots with acetocarmine. First germinate cabbage seed on water agar or moist filter paper until the root is about 2 cm long. Then put the root of the seedling through a small hole made in aluminum foil that covers small beakers or vials containing a suspension of purified resting sporangia, at a concentration of 10^5-10^7/ml, in quarter-strength Hoagland's nutrient solution. The base of the vials should be kept darkened by wrapping the vial in aluminum foil. Several young plants can be grown in a single beaker by puncturing numerous holes through the foil cover. Plants may be grown in the laboratory under artificial light or in a window. A few seedlings should be removed periodically after 2-3 days and root hairs examined under a phase-contrast or dark-field microscope for plasmodia or secondary sporangia. Whole seedlings or their root systems may be boiled for 5 min in acetocarmine or left overnight in the stain at room temperature and then examined with the light microscope for plasmodia in the root hairs. Plasmodia will appear as densely red, oblong to oval clumps of cytoplasm in a lightly stained root hair. Secondary zoosporangia appear as clusters of deeply staining spheres in the root hair. When root hairs are infected at an early stage, considerable distortion of the elongating root hair occurs. Often the tip of the hair will be swollen and club-shaped or branched. If the progress of root hair infection is watched daily for over a week, secondary zoospores may often be observed swimming actively within the root hair cells. These zoospores are thought to be gametes of the organism and to arise from the secondary sporangia.

Biological Phenomena

Biological Races Numerous biological or host specialized races of *P. brassicae* exist. In the United States the most widespread is Race 6, which occurs on members of *Brassica oleracea* (cole crops) but does not attack *Brassica napobrassica* (rutabaga or swede). In eastern Canada and Europe, races that attack *B. napobrassica* are common (Williams, 1966). A set of four differential crucifers has been devised for assaying isolates of *P. brassicae* and categorizing them on the basis of their reaction on the differential hosts. The four host plants and sources of seed are listed below:

- *Brassica oleracea capitata* cv. Jersey Queen (cabbage), Olds Seed Co., Madison, Wis.
- *Brassica oleracea capitata* cv. Badger Shipper (cabbage), P. H. Williams, Univ. Wisconsin, Madison, Wis. 53706
- *Brassica napobrassica* cv. Laurentian (rutabaga), Olds Seed Co., Madison, Wis.
- *Brassica napobrassica* cv. Wilhelmsburger (rutabaga) Dr. George Ayers, Canada Department of Agriculture Research Station, Charlottetown, Prince Edward Island, Canada.

When various isolates of clubroot have been collected, spores should be purified as described previously and 25 seedlings of each differential transplanted into each batch of soil containing a separate spore isolate or collection. Grow plants in pots (5/pot) for a month; then dig and examine for clubroot. Depending on the reaction of the various isolates to the four differential hosts, the isolates can be classified into race classes as follows (Table I).

Since races that attack rutabaga are uncommon in the United States, they may be obtained from Dr. George Ayers listed above. Great care should be exercised in sterilizing all soil, pots, flats, and pans in which soil is mixed after the experiments are finished. Clubroot persists for many years in the soil and may be a potential hazard to crucifer growers in the area.

Host Range Different crucifer weed and cultivated species can be transplanted into infested soil and the

TABLE 1 Possible Host Reactions to Infection by Races of *Plasmodiophora brassicae**

Differential	Race															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Cabbage:																
Jersey Queen	+	+	+	+	-	+	+	-	-	+	-	+	-	-	-	-
Badger Shipper	-	+	-	+	-	-	+	-	-	+	+	-	+	+	+	-
Rutabaga:																
Laurentian	+	+	+	+	-	-	-	+	+	-	+	-	+	-	-	-
Wilhelmsburger	+	-	-	+	-	-	-	-	+	+	+	+	-	+	-	+

* + indicates a susceptible host reaction;
 - indicates a resistant host reaction.

spectrum of susceptibility surveyed; some crucifers will be resistant whereas others will be highly susceptible. Others will develop galls of varying morphology, from round nodular types to long spindle-shapes ones.

9 Trichomycetes

OCCURRENCE

Table 2 lists representative host genera, their common names and types of habitat. The list is by no means complete and is restricted to hosts that can be commonly found in North America. Examination of reference material will provide additional listings. Most institutions in North America are within a reasonable collecting distance of suitable trichomycete hosts. Though the appearance of many arthropods is seasonal, particularly where winters are severe, it is possible in many areas to collect throughout the year from known populations of such hosts as black fly and midge larvae, millipeds that burrow in logs or inhabit greenhouses, and a wide variety of intertidal arthropods.

The endozooic trichomycete thalli are usually firmly attached by means of a holdfast to the chitinous lining of the host gut. Most genera are restricted to the hindgut, a few to the midgut (attached to the peritrophic membrane)--some are known only from the foregut or from the foregut and hindgut. The single ectozooic genus *Amoebidium* occurs on various external parts of aquatic arthropods. Since the chitinous linings of the gut are ectodermal in origin, they are shed along with the exoskeleton when the host molts. Newly molted specimens, therefore, would not ordinarily contain thalli of Trichomycetes. Fresh molts (which can often

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TABLE 2 Representative Arthropod Hosts of Trichomycetes

Classification	Representative Genera	Common Name	Habitat
Class CRUSTACEA			
Decapoda (Anomura)	*Callianassa*	ghost shrimp	mud flats or tide zone
	Emerita	mole crab	tide zone
	Eupagurus	hermit crab	tide zone
	Pagurus	hermit crab	tide zone
	Petrolisthes	porcelain crab	tide zone
	Upogebia	mud shrimp	mud flats
(Brachyura)	*Hemigrapsus*	shore crab	tide zone
	Sesarma	marsh crab	tide zone
	Uca	fiddler crab	tide zone
(Astacura)	*Orconectes*	crayfish	streams and ponds
Cladocera	*Daphnia*	water flea	ponds
Isopoda	*Armadillidium*	pillbug	terrestrial
	Asellus	sowbug	fresh water
	Ligia	rock louse	splash zone
	Porcellio	sowbug	terrestrial
	Sphaeroma	pillbug	tide zone
Amphipoda	*Orchestia*	sand flea	high tide zone
	Orchestoidea	beach hopper	splash or high tide zone
	Talorchestia	sand flea	high tide zone
Class DIPLOPODA			
Polydesmida	*Apheloria*	milliped	terrestrial
	Boraria	milliped	terrestrial
	Dixidesmus	milliped	terrestrial
	Euryurus	milliped	terrestrial
	Oxidus	milliped	terrestrial
	Scytonotus	milliped	terrestrial
Spirobolida	*Californibolus*	milliped	terrestrial
	Narceus (Spirobolus)	milliped	terrestrial
Class INSECTA			
Ephemerida (Plectoptera)	*Callibaetis*	mayfly (nymphs)	ponds and streams
Coleoptera	*Popilius (Passalus)*	passalid beetle	terrestrial
Diptera (Simuliidae)	*Simulium*	black fly (larvae)	small streams
(Chironomidae)	*Chironomus*	midge (larvae	ponds, pools, and streams
(Culicidae)	*Aedes*	mosquito (larvae)	ponds and pools
	Culiseta	mosquito (larvae)	ponds and pools
	Culex	mosquito (larvae)	ponds and pools

be found in cultures of hosts in the laboratory) should be collected and examined. In the molts it is sometimes possible to find reproductive stages of the fungi that are not abundant in unmolted host specimens.

Among the best sources of Trichomycetes are larvae of Diptera. Such genera as *Simulium* or *Chironomus* frequently are infected (infested) with more than one species of fungus. Milliped populations may be a reliable source for the genus *Enterobryus*, and those who live near the seashore may find a continuous supply of Eccrinales in crabs, particularly crabs that burrow in mud.

The presence and abundance of Trichomycetes is dependent upon many factors. In general, arthropods that contain the fungi are those that are scavengers or eat such plant material as algae or decaying leaves and wood. The strictly predaceous or carnivorous types are not likely to be infected. Populations of the same host species that occupy different geographical habitats may vary considerably in the percentage of individuals with Trichomycetes. The percentage of infected individuals is apt to be greatest in those species that live in dense communities, such as the larvae of some mosquitoes, black flies, and midges, the adults of various isopods and amphipods, communal millipeds, and gregarious decapods like the fiddler crab (*Uca*) and shore crab (*Hemigrapsus*).

An instructor intending to use new or unfamiliar arthropods in class would do well to dissect a number of specimens before turning them over to the class. While the discovery of these peculiar fungi can be a stimulating experience to students, dissecting many arthropods without reward can have quite the opposite effect.

COLLECTION AND MAINTENANCE

In collecting arthropods it is well initially to solicit the advice of an entomologist or invertebrate zoologist familiar with the local fauna. There are also a few good illustrated books available for the nonspecialist. Morgan (1930) gives many helpful details on the collection of arthropods in ponds and streams. J. W. Hedgpeth's revision of Ricketts and Calvin (1952) has a wealth of information about intertidal arthropods of the Pacific coast. For the Atlantic coast, Miner's book (1950) can be very useful. Other references may be obtained for information on particular areas or types of hosts.

Streams and Ponds

One of the handiest pieces of equipment for collecting in aquatic environments is an inexpensive 6-inch metal round-bottomed food or tea strainer, with the two prongs opposite the handle bent backward against the strainer. It is sturdy enough to be scraped over rocky bottoms and dragged through sand, mud, and weeds, and can also be used to catch insects that are swept downstream from disturbed stones or scraped from rocks in fast-flowing waters. A smaller strainer (3 in) may be useful for more selective collecting in small pools. The animals thus caught can be picked from the strainer screen with forceps, or they can be knocked directly into a jar or pail by tapping the strainer against the mouth of the container.

In streams, various arthropod hosts can be picked directly from rocks lifted out of the water. Mayfly and stonefly nymphs can be found scurrying on the undersurface of such rocks. Stones covered with algae may harbor various midge larvae. Black fly larvae are more or less restricted to riffles and other rapidly-flowing water, where they attach themselves to rocks or submerged border vegetation.

Living and dead vegetation in ponds may yield amphipods, isopods, mayfly nymphs, and midge larvae. The latter may form soft tubes on vegetation or burrow in the mud. *Chironomus* larvae, known as bloodworms because of their color, are easily identified and often are larger than other midge larvae. Mosquito larvae in small pools should be sought. Plankton nets may be used to collect minute Crustacea.

It is not advisable to use insect repellents or insecticides on the hands when collecting in aquatic environments.

Terrestrial Habitats

Woodlands are generally the best source for terrestrial hosts, particularly where fallen and decaying timber exists and molding deciduous leaves are plentiful. Millipeds and pillbugs may be collected in leaf litter, from under logs, beneath dead bark, or inside rotting wood. Of the many genera of millipeds in North America, species of the orders Polydesmida and Spirobolida are most apt to be infected with Trichomycetes. One may find the large passalid beetle living communally in tunnels in decaying wood. These are usually infected with *Enterobryus attenuatus*. (Incidentally, the beetles and their associated mites are usually also infected with the Laboulbeniales genus *Rickia*.) A long-bladed hunting knife is a useful tool for tearing open logs as well as for raking about in leaf mold.

Marine Habitats

Most marine hosts of Trichomycetes are intertidal. Therefore, low tide is best for collecting. Equipment needs are simple: cotton gloves, a hand trowel, and pails usually suffice. Hermit crabs may be found in abundance in tide pools or other shallow waters, but the percentage of infected individuals is generally low. Porcelain crabs and isopods can be found under rocks near the high-tide zone. Mud flats may have crabs such as *Uca* or *Hemigrapsus* infected with *Enterobryus* or *Taeniella*, and if one has a shovel and a strong back, anomurids such as *Callianassa* and *Upogebia* may be collected and their foregut (stomach) examined for *Enteromyces*.

Mole or sand crabs (*Emerita*) prefer sandy beaches where breakers occur. Beaches with washed-up seaweeds and other rubbish generally have a rich fauna of beach hoppers or sand fleas. The agile rock louse (*Ligia*) runs about on cliffs, large rocks, and pilings, and can be caught by flipping specimens into a pail using a hand trowel or knife.

Transporting Specimens

Because preserved host specimens are not satisfactory for dissections, it is desirable to keep the animals alive. With many arthropods this presents little difficulty, but some types may die within hours if improperly handled, especially in hot weather.

Suitable containers include glass or plastic jars, plastic bags, and pails. Handy containers can be made from Mason jars (pint, half-pint, and quart sizes, preferably wide-mouth) by cutting out a circle of aluminum screening and inserting this in the band that screws to the jar. The screen should be slightly larger than the diameter of the band so as to be retained firmly in place. The metal disk that ordinarily serves as the lid of the jar can be inserted when desired (without removing the screen) to made a water-tight container.

Aquatic arthropods from ponds or pools may be transported in shallow pond water with the possible addition of some plant material, some mud, etc., to suit individual needs. Hosts from rapidly-flowing waters, such as *Simulium* larvae, generally must be kept in shallow layers of water to provide adequate aeration, and, if transportation distances are great, it is advisable to place them in an ice chest.

Terrestrial arthropods survive readily in containers that are not sealed. Crowding should generally be avoided, and moist leaf litter or decaying wood should be included. If these arthropods are to be maintained in terraria for some time, it is advisable to fill large plastic bags with additional moldy leaves and wood from near the collection site, since substratum obtained near cultivated or urban areas may contain insecticides. Collections of some millipeds, such as *Narceus (Spirobolus)* and *Apheloria*, should be handled in such a way that the animals are not permitted excessive contact with each other, and they should not be handled too roughly; products secreted from the glands of some millipeds may prove fatal to confined specimens.

Marine arthropods should be transported in very shallow sea water in pails or other containers such as plastic bags. Increases in salinity due to evaporation should be avoided by occasionally adding fresh water or by periodically changing the sea water.

Maintenance in the Laboratory

Freshwater arthropods can be kept in aquaria for varying lengths of time, though here again it is best if they are refrigerated and used within a few days after collecting. Those from quiet waters, such as mosquitoes and some midge larvae, survive well and will often pupate and emerge as adults (these latter can be retained for identification purposes). Except for mosquito larvae, it is best to keep the animals in shallow water to which may be added some plant material or mud, depending on the nature of the original environment.

Black fly larvae can most conveniently be kept alive by transferring them to containers (Petri dishes, or any wide container with a lid), covering them with a shallow layer of distilled or stream water, and placing them in a refrigerator. In this way they may be kept for several days to several weeks. Other types of arthropods from fast-flowing waters can be handled in a like manner.

Collections from terrestrial environments can usually be kept for long periods of time in covered terraria with an abundance of decaying leaves and wood, provided the animals are not overcrowded and moisture conditions are satisfactory. Overwatering is to be avoided; occasional sprinkling of the surface to maintain moisture is usually adequate. Isopods and some millipeds breed well in terraria, so that a continual supply of infected hosts may be provided. Mixtures of different arthropods can usually be kept successfully in a single large terrarium.

Marine hosts vary in the ease with which they can be maintained outside of their natural habitat. It is best to use them soon after collecting, unless running sea water or aerating and filtering equipment are available. Refrigerating most marine hosts in a minimum of sea water will keep them alive for many days. Some marine arthropods are very hardy; specimens of *Uca* and *Sphaeroma*, for example, have been kept alive in inland laboratories for over a year in aerated marine aquaria, fed with small amounts of rolled oats.

Though overcrowding is generally to be avoided, species that can live in nature in dense populations and can survive well in the laboratory (e.g., mosquito larvae, pillbugs), if maintained under crowded conditions, often show an increase in the proportion of infected individuals.

DISSECTION AND PREPARATION OF MATERIAL

The most valuable tools for dissection are two pairs of fine-pointed jewelers' forceps (sizes H3 and H2 are good for most specimens). A pair of fine iris scissors is also desirable. Extra fine dissecting needles can be made from "minuten" insect needles mounted in wooden or glass holders.

Dissections should be carried out under a good dissecting microscope with adequate illumination. Reflected light against a dark background is generally satisfactory. Magnifications from 6 to 30X may be used, depending upon the size of the host; somewhat higher magnifications may be useful for examining opened guts. The animals can be dissected alive, or they may first be mechanically or chemically inactivated.

The hindguts of immature insects, adult beetles, isopods and amphipods can usually be removed quickly and easily by holding the animal with one pair of forceps and grasping the region of the anus with another pair. By pulling firmly but slowly, one can remove the intact hindgut, which usually breaks away from the midgut where the two structures meet.

The peritrophic membrane of infected hosts is a transparent, chitinous tube present in the region of the midgut and possibly extending a distance into the hindgut cavity. Unlike the chitinous linings of the hind- and foreguts, it is not fused to the epithelium but is attached only near the anterior end of the midgut. In some aquatic insect larvae the peritrophic membrane is well developed, and one may find the unbranched thalli of the Harpellaceae attached to it. The membrane might be drawn from the insect body when the hindgut is pulled, or it may be recovered separately. It is sometimes packed with algal cells and debris, which can be removed by simply lifting the membrane several times from the water in which it is dissected, allowing the surface tension of the water to squeeze out all unattached material. It need not be cut open for mounting.

The abdomen of crabs is folded under the thorax. Removal of the abdomen by cutting or tearing will bring with it the hindgut. The gut can then be separated from the abdomen by making two longitudinal ventral cuts into the abdomen on each side of the gut.

The hindgut of decapods with extended abdomens may be dissected out by making parallel cuts, one on each side of a median dorsal line. The foregut (stomach) of some decapods (*Uca*, *Callianassa*, *Upogebia*, hermit crabs, and crayfish) may contain species of Eccrinales. This organ can best be removed by dissection from the dorsal surface.

Milliped hindguts can be dissected out by cutting off the anterior and posterior segments of the body and then pulling out the hindgut from the posterior end.

Larger guts can be cut open with scissors. For smaller guts a fine needle may be satisfactory for making the incision. Extremely small guts, such as those from some dipterous larvae, may be opened by careful tearing with two fine-pointed forceps.

The dissection of guts should be made in water. Distilled water is satisfactory in most instances. In rare cases, such as *Asellaria* from *Ligia*, cells may rupture, so that it is safer to use a saline solution. If saline solutions or sea water are used, care should be taken to prevent the salts from becoming concentrated due to evaporation.

After the guts are opened, flood with distilled water (or saline, etc.), using a fine jet of water from a bulb, to dislodge the contents of the gut. Any Trichomycetes present will remain attached by their holdfasts. If the fungal thalli are abundant or long (some thalli of Eccrinales may measure up to 1 cm or more), they may be easily visible under low magnification or with the unaided eye. However, the thalli of many Trichomycetes are not so obvious to the untrained observer, and the guts should be examined carefully under higher magnification before being discarded. (The examination may be delayed until the epithelium is removed, as indicated below.) With a minimum of experience it is possible to distinguish the trichomycete material from such host structures as Malpighian tubes (located at the juncture of the hindgut and midgut), tracheae, and muscle fibers.

If the lining, or parts of it, are to be mounted on a slide, it is necessary to remove the epithelial tissue to which the transparent chitinous lining is attached. In some cases this can be done by carefully separating the two structures in water. With many dipteran larvae, the unopened gut can be held at one end and gently pulled through a pair of fine forceps, leaving the epithelium behind. The chitinous lining of arthropods may become loosened from the epithelium if the specimens have been dead for many hours. Such material can be used, provided one is alert to the possibility that artifacts may occur in the trichomycete thalli.

A simple technique for loosening the gut lining is to soak the opened and washed gut in dilute lactophenol (approximately one drop of lactophenol per ml of water) for a few minutes to many hours, depending upon the material. The lining will usually come loose easily, and after being rinsed in clean dilute lactophenol, can be mounted in a drop of regular strength lactophenol with cotton blue, and sealed.

Water mounts may be preferred if living material is to be observed with a phase contrast or ordinary light microscope. If this is the case, it is necessary to mount pieces of the gut lining, or thalli removed from the lining, without resorting to the dilute lactophenol treatment. Water mounts on slides may be kept in moist chambers for days, and stages of vegetative growth and maturation and release of spores may be observed in some species, particularly in members of the Harpellales and Amoebidiales.

Various standard fixatives, stains, and other mounting techniques can be used, according to preference and depending upon the studies to be carried out.

CULTURING

A most significant development in recent years has been the axenic culturing of Trichomycetes (Whisler, 1962; Clark *et al.*, 1963; Lichtwardt, 1964). To date, only species of *Amoebidium* and *Smittium* have been cultured, but of these there are more than 70 isolates (representing *Amoebidium parasiticum* and five species of *Smittium*) from various hosts over a wide geographical range.

The nutritional studies on *Amoebidium* by Whisler (1962) should be consulted. He found that maximum growth occurred on a thiamine-enriched tryptone-glucose medium with salts but that methionine could replace tryptone to give a defined medium.

Growth requirements of *Smittium* spp. are currently under investigation, but, in general, they can be grown on a variety of natural or semi-synthetic media and also on defined media such as methionine-glucose.

Cultures of *Smittium culisetae* and *S. simulii* from mosquito and black fly larvae, respectively (Lichtwardt, 1964), are available from the American Type Culture Collection or from the Department of Botany, the University of Kansas. *Smittium culisetae* grows more vigorously and is to be preferred for class use. Cultures of *Amoebidium parasiticum* also may be obtained from the University of Kansas.

Smittium can be cultured in liquid or agar media. If agar is used, the medium should be flooded with a thin overlayer of sterile, distilled water before inoculation. Among the media that might be used, the following two are recommended--M-1 and M-56.

The medium in Petri dishes should be inoculated from young cultures, using a small loop to transfer pieces of colonies. The mycelium should then be broken up on the plate. Fairly heavy inoculation is desirable. Within 5-7 days at room temperature there should be noticeable growth and abundant formation of spores.

Amoebidium may be grown on the same kinds of media. Mycelium is not produced in this genus, so the inoculum should consist of a loopful of thalli and spores from a young culture.

Species of these culturable fungi can be maintained at room temperature on test tube slants containing 1 or 2 ml of distilled water. After inoculation the tubes should be tipped daily for a few days to allow the water at the bottom of the tubes to run over the slanted medium. Transfers should be made frequently, preferably every 2-3 months.

Isolations of *Smittium* (or *Amoebidium*) can now be made rather routinely. Many methods and media have been tried, and a number of these have proven successful. The method described below has given good results rather consistently.

Pieces of the gut lining with attached thalli of *Smittium* (or pieces of integument with *Amoebidium*) should be dissected out in a drop of water, washed three or four times consecutively in small vessels containing sterile water and antibiotics, and plated in small Petri dishes containing a medium with an overlayer of sterile water and antibiotics as described below. Transferring may be done with a very small, fine-metal loop, using aseptic techniques.

It is convenient to use 35 x 10 mm plastic disposable Petri dishes for washing, and 60 x 15 mm plastic dishes for the initial plating. The latter should have very smooth (unwrinkled) tops to permit viewing the material under a 50X objective for possible contamination by bacteria, yeasts, or molds. If contaminants are recognized early, it is often possible to save the trichomycete thalli by rewashing and replating; if bacteria are the contaminants, more concentrated antibiotics may be used, although resistant bacteria may pose a problem in some isolations.

A mixture of penicillin and streptomycin (40,000 units penicillin and 80,000 units streptomycin per ml of water) can be prepared and sterilized by filtration, and 0.1-0.2 ml of this solution can be added to each wash dish and to the water overlayer in the dish of medium. Concentrations of up to 0.5 ml per dish have been used without apparent deleterious effects upon the trichomycete thalli.

Various isolation media have been used successfully, but one of the best seems to be dilute (10%) brain-heart infusion agar (M-1). If growth occurs, it generally becomes evident under the microscope within 2-5 days. Subcultures should be made as soon as practicable.

MORPHOLOGICAL FEATURES OF COMMON GENERA

The following is a simplified key to the major families of Trichomycetes:

1. Spores produced internally; thalli usually unbranched 2
    2. No amoeboid cells produced; spores usually produced singly in terminal series of sporangia (ECCRINALES) 3
        3. Cells (sporangia) do not germinate *in situ* . . . . . . ECCRINACEAE
        3. Cells (sporangia) may germinate *in situ* to produce lateral filaments. . PALAVASCIACEAE
    2. Capable of producing amoeboid cells; entire thallus functions as one sporangium (AMOEBIDIALES). . . . . AMOEBIDIACEAE
1. Spores produced externally or by fragmentation of branches 4
    4. Thalli branched, reproducing by fragmentation (arthrospores) ASELLARIALES. . . . . . ASELLARIACEAE
    4. Thalli simple or branched; spores exogenous (HARPELLALES). 5
        5. Thalli simple . . . . HARPELLACEAE
        5. Thalli branched . . . . . GENISTELLACEAE

Table 3 is a partial list of genera that may be found in North America. Some of the fairly common North American genera are not listed because the genera or their species have not yet been described.

For additional information, see Duboscq, 1948; Lichtwardt, 1954, 1957a, 1957b, 1958, 1960a, 1960b, 1962; Whisler, 1963; Manier and Lichtwardt, 1968; Manier, 1969.

TABLE 3 Selected Genera of Trichomycetes

Genus	Location in Host	Types of Hosts
Eccrinales		
ECCRINACEAE		
Enterobryus Leidy	hindgut (and foregut)	millipeds, beetles, crabs
Arundinula Léger and Duboscq	foregut and hindgut	Crustacea (hermit crabs, crayfish)
Enteromyces Lichtwardt	foregut	ghost shrimps, crabs
PALAVASCIACEAE		
Palavascia Tuzet and Manier	hindgut	intertidal Isopods (*Sphaeroma)*
PARATAENIELLACEAE		
Parataeniella Poisson	hindgut	Isopods (pillbugs, sowbugs)
Amoebidiales		
AMOEBIDIACEAE		
Amoebidium Cienkowski	external	minute aquatic Crustacea and various immature aquatic insects
Paramoebidium Léger and Duboscq	hindgut	aquatic Diptera larvae; mayfly nymphs
Asellariales		
ASELLARIACEAE		
Asellaria Poisson	hindgut	intertidal and terrestrial Isopods (*Ligia, Armadillidium)*
Harpellales		
HARPELLACEAE		
Harpella Léger and Duboscq	midgut (peritrophic membrane)	black fly larvae
Stachylina Léger and Gauthier	midgut (peritrophic membrane)	midge larvae
GENISTELLACEAE		
Smittium (Rubetella) Poisson	hindgut	Diptera larvae (black flies, mosquitoes, midges)
Pteromaktron Whisler	hindgut	mayfly nymphs

10 Hemiascomycetes

PROTOMYCETACEAE

All members of the Protomycetaceae are specialized plant parasites, and most of their life cycle is completed inside the host plant. Classification of the family has not been easy partly because of the difficulty in acquiring sufficient cytological information. However, *Protomyces inundatus*, parasitic on *Apium nodiflorum*, has been intensively investigated and has proved to be excellent material for illustrating the main features of the family. Unlike other members it completes its life cycle in a relatively short time and so is suitable for class purposes.

Protomyces macrosporus on *Aegopodium podagraria* and *P. pachydermus* on *Taraxacum officinale* are also fairly common, in Great Britain at least. These are as host specific as is *P. inundatus*. The same conditions for infection are necessary and galls appear in about 14 days after inoculation. Inoculations in the field to show host specificity can be carried out as follows: a tin box, approximately 2 ft in each dimension, with a glass lid, is fitted around either *Taraxacum officinale* or *Aegopodium podagraria* plants and host leaves and stems infected with *P. pachydermus* or *P. macrosporus* tied to the uninfected host at about October. In April or May, the following year, the *Taraxacum* plants associated with *P. pachydermus* inoculum will show infection, as will the *Aegopodium* with *P. macrosporus*, but no other combinations will do so.

For additional information, see Tubaki, 1957; Valadon, 1963, 1964; Valadon *et al.*, 1962a,b.

Cultural Isolates

Haploid and diploid cultures can be obtained from chlamydospores. Mature chlamydospores are removed from the host and surface sterilized by washing in 0.1% mercuric chloride for 5 min, rinsing in distilled water and washing under running water for 1 hour. The chlamydospores are then squashed in sterile distilled water to release the endospores. A drop of endospore suspension is transferred, using a sterile Pasteur pipette, to a slide covered with a thin layer of sterile 2% malt extract agar. The slide is tilted to spread the suspension and then left until the water disappears. The slide is now placed on a moving stage of a microscope, and by using a dummy objective individual endospores can be picked up and transferred to culture medium. It is possible to distinguish between unfused (haploid) and fused (diploid) endospores and therefore to obtain cultures derived from either or both of them. Cultures derived from both fused and unfused endospores look alike--they consist of colonies of yeast-like budding cells; there is, however, a small size difference in the cells--they average $55.43\mu^3$ and $53.21\mu^3$, respectively.

Cytology of Protomyces inundatus

P. inundatus will grow, but does not complete its life cycle, in culture. It will grow on solid and liquid medium; it grows well on Lilly & Barnett's semi-synthetic medium and will also grow on 2% malt extract medium; it has a requirement for biotin and thiamine.

Cultures can be made from a) single endospores, which give rise to colonies of haploid yeast-like budding cells, or b) fused endospores, which give rise to colonies of diploid yeast-like budding cells, or c) mycelium from infected host tissue, which also gives rise to colonies of diploid yeast-like budding cells.

The nuclei of endospores can be demonstrated using acetoorcein (SR-9), in the following manner:

- Fix endospores from about 20 chlamydospores in 1 ml acetic alcohol (1:6) for 24 hours. Place a drop of the mixture on a slide and cover with a cover slip; remove excess fluid by pressing between several layers of filter paper.
- Place slide on dry ice for 2 min and then remove cover slip using a sharp scalpel; plunge slide into absolute alcohol and leave for 24 hours.
- Now transfer to 70% alcohol and leave for 30 min; place in cold 1.0 N HCl for 30 min and transfer to 1.0 N HCl at 60°C and leave for 8-12 min.
- Rinse in distilled water and then in 45% acetic acid, remove excess fluid, and place a drop of aceto-orcein on slide, cover with a cover slip and seal with Waller's cement.

Unfused endospores (5-8μ long) are uninucleate, while the conjugated ones sometimes contain two nuclei, sometimes one. The two nuclei of the conjugated endospores occur in various positions, but a series can usually be obtained in which the two nuclei are progressively nearer the bridge of the conjugated endospores, where they eventually fuse. The nuclei are very small, and it is impossible to differentiate between haploid and diploid ones.

Experimental Infection of Host

The host, *Apium nodiflorum*, is widely distributed, at least in Great Britain, near or in streams. It is easily established in pots in the greenhouse at a temperature of about 20°C.

To infect the host plant, use 14-day-old cultures, derived from fused endospores, on 2% malt agar slants. Suspend the diploid cells in sterile distilled water and paint the suspension onto the host. Then cover the plants with cylindrical polythene-covered cases with muslin tops, thus ensuring a high relative humidity around the plant. After 14 days swellings and galls

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appear on the inoculated leaves and stems. The greenhouse temperature must be kept at or below 20°C to ensure maximum infection.

Similar inoculations using cultures derived from single unfused endospores, i.e. haploid cells, do not result in infection of the host.

Demonstration of Heterothallism

P. inundatus is heterothallic and the life cycle can be completed only if two strains are present, as the following experiments demonstrate.

On Host From several haploid cultures, derived from single unfused endospores and grown on 2% malt extract agar slants, make cell suspensions for inoculating host plants. No one of these haploid cultures will cause infection, but if inoculated in pairs, certain combinations lead to infection.

A cross-inoculation experiment, using cultures from four endospores, might give results as shown below, in which a plus designates infection and a minus designates no infection. In the example shown, cultures #1 and #2, #1 and #3, #2 and #4, and #3 and #4 produce infection. All other combinations do not.

Culture	#1	#2	#3	#4
#1	-	+	+	-
#2	+	-	-	+
#3	+	-	-	+
#4	-	+	+	-

In Culture Two haploid cultures may be crossed by streaking the cultures side by side on 2% malt agar and letting them grow for 7 days. Then mix the two colonies, using a sterile needle, and after 24 hours 60-70% conjugated endospores may be seen if the initial two cultures were of the opposite strain. If the two cultures were of the same strain, no fused endospores will be seen.

Demonstration of Ascospores

Ascospores can be produced on a number of species of yeast, as summarized below. All of the yeast cultures here considered can be stored for at least a year on malt extract agar (M-38) slants under sterile mineral oil (heavy liquid petrolatum #9), with the exception of *Eremascus albus*, an osmophilic species that does not grow on ordinary malt extract agar and should be transferred, as well as stored, on osmotic medium.

For storage of yeast cultures, transfer to fresh stock medium. After growth has occurred (3-5 days), sterile mineral oil is poured from a test tube onto the slant culture to a level about 1 cm above the thin end of the agar. Cultures should be stored in a cool place (e.g. 18-20°C), but not in a refrigerator.

At least three weeks before class use, the yeasts should be transferred from storage to fresh malt agar slants, as some yeasts grow slowly when first transferred from under oil. Incubate at room temperature.

To achieve sporulation, transfer one loopful of yeast to sporulation medium from a 24-hour culture on malt extract agar, and incubate at room temperature. A given culture may vary from year to year with respect to optimal sporulation results. Asci and spores are best observed by suspending a small amount of the growth on the slant in a droplet of water. Sample the slant in various places and take only surface growth, since in the most aerobic portion one generally finds the greatest number of spores.

For additional information on sporulation in yeast, see Phaff and Mrak, 1948, 1949; Miller and Hoffman-Ostenhof, 1964.

Dipodascus uninucleatus This haploid species sporulates well on melibiose medium (M-72) in 5-7 days and on malt extract agar (M-38) in 8-10 days. In 5 days good examples of immature asci can be seen. Neighboring cells of the mycelium conjugate through short protuberances; after fusion is complete, the fusion product develops into a large conical ascus. In water mounts spore discharge can be observed in fully sporulated cultures.

Eremascus albus This haploid species sporulates on osmotic medium (M-68) in 14-20 days, but at 10 days good examples of conjugation stages and immature asci can be seen. After conjugation, the fusion product is separated from the hyphae by the formation of septa and develops into a spherical ascus with eight ovoidal or spheroidal spores.

Endomyces reessii This haploid species sporulates on vegetable agar (M-32) or on malt extract agar (M-38) in 5-7 days. Younger cultures may be observed for structures that develop into asci. The spores are prolate-ellipsoidal, have a thick wall surrounded by a thin creased membrane, and contain a large lipid globule.

Nadsonia elongata Sporulates well on malt extract agar (M-38) in 2-5 days. In this genus conjugation (plasmogamy) occurs between a large mother cell and its smaller bud; the zygote then forms a bud at the end opposite to the first bud and the contents of the zygote move into the second bud, which is transformed into the ascus. Usually one, but occasionally two, spherical spores with a spiny surface are formed that have a pronounced oil globule in the center. The color is dark, giving sporulating cultures a brownish appearance.

Schizosaccharomyces octosporus This haploid species sporulates on osmotic medium (M-68) and on malt extract agar (M-38) in 2-5 days. Asci are much larger than the vegetative cells, usually dumbell-shaped, but the central constriction is not very pronounced. There are usually eight ovoidal ascospores per ascus; there is a delayed liberation of the spores from the ascus upon maturity.

Schizosaccharomyces pombe This haploid species sporulates on osmotic medium (M-68) and on malt extract agar (M-38) in 5-7 days. Asci result from the fusion of two haploid cells and are often somewhat curved, usually containing four ovoidal ascospores. Liberation of the ascospores from the mature ascus is delayed.

Endomycopsis capsularis This haploid species sporulates on carrot wedges (M-3) or on malt extract agar (M-38) in 4-7 days. Carrot wedges should be inoculated on the top third of the wedge with a small amount of yeast. In this way growth proceeds to the lower portion and yeast at different ages can be sampled. The asci are formed at the tips of the hyphae or are intercalary. No clear evidence of conjugation is visible. The spores are spheroidal to ovoidal, occasionally showing a subequatorial ridge. Four spores are usually formed in each ascus and are rapidly liberated when the asci are mature.

Saccharomyces cerevisiae This diploid species sporulates well on acetate agar (M-57) in 4-7 days. Asci are ovoidal or globose; there are two to four spheroidal or globose spores per ascus; asci do not rupture at maturity.

Saccharomyces acidifaciens This haploid species sporulates on malt extract agar (M-38) in 4-7 days. On younger slants (about 2 days old) zygotes (dumbell-shaped immature asci) can be seen. Asci remain dumbell-shaped; there are two to four spheroidal or globose spores per ascus; asci do not rupture at maturity.

Saccharomyces fragilis This diploid yeast usually sporulates abundantly on malt extract agar (M-38) in 3-6 days. The asci are elongate, even cylindrical, in shape. The ascus wall is very thin and the asci lyse very soon after the spores are mature, thus liberating them. The four spores are kidney-, bean-, or crescent-shaped, often linearly arranged in each ascus.

Hansenula anomala This diploid species sporulates well on malt extract agar (M-38) after 2-5 days. The asci are ovoidal to elongate and contain 2-4 spores, shaped like derby hats with a pronounced brim. The asci rupture easily upon maturity.

Schwanniomyces alluvius This haploid species sporulates best on yeast autolysate glucose agar (M-60) in 7-10 days. The asci are globose vegetative cells that contain a smaller bud-like structure attached by a rather wide neck wherein karyogamy and meiosis occur. The spores, one or more rarely two, are finally formed in the mother cell. The spores have a large oil globule, a pronounced equatorial ridge and a somewhat warty or wrinkled surface, giving them the appearance of a walnut. Asci do not rupture at maturity.

Debaryomyces hansenii This haploid species sporulates best on Gorodkowa agar (M-49) after 3-5 days. The asci may result from conjugation with a bud (heterogamic conjugation) or from conjugation with another somatic cell (isogamic conjugation), and usually contain one and more rarely two spheroidal spores with a small lipid globule and a more or less warty wall. Asci do not rupture at maturity.

Hanseniaspora valbyensis This diploid species sporulates abundantly on malt extract agar (M-38) in 1-3 days. Asci are apiculate (lemon-shaped) or ovoidal and contain four small hemispheroidal or hat-shaped spores with a very narrow brim. The asci rupture very readily upon maturity, often resulting in large masses or clumps of liberated spores.

Nematospora coryli This yeast sporulates on V-8 juice agar (M-30) in 3-7 days; timing is somewhat erratic. Prior to sporulation, a cell enlarges and becomes elongate to cylindrical with rounded ends. Eight needle-shaped ascospores then develop in the ascus. Spores are sharply pointed at one end and have a long whip-like appendage at the other end. Asci rupture at maturity.

For additional information, see Phaff and Mrak, 1948, 1949; Miller and Hoffmann-Ostenhof, 1964.

Isolation of Lipomyces from Soil

Species of *Lipomyces* have thus far been isolated only from various soils, although the yeast is more abundant in some than in others. It is recommended that collections be made from meadow, cultivated farm land, fallow land, forest, orchard, garden, desert, and so on. Soil yeasts are generally most abundant some 5 cm below the surface, where the cells are less subject to desiccation, high temperatures, and radiation. A shady area usually yields more yeast than does a fully exposed one.

When moderately fine, dry soil particles are scattered on a plate of nitrogen-free mineral medium (M-84) and incubated at room temperature, cells of *Lipomyces* often develop into slimy, watery colonies around a soil particle. The particles should be separate from each other. Wet soils should be allowed to dry out and large clumps crushed aseptically. The cells of such colonies are highly capsulated and contain large fat globules. It is not clear why *Lipomyces* is often found in this way, since there is no evidence that this species is able to fix atmospheric nitrogen. Presumably traces of nitrogen in the soil particle, or growth of nitrogen-fixing *Azotobacter* cells, permit the yeast to develop.

Azotobacter usually develops first (2-3 days) around the soil particles and is characterized by glistening, soft, smooth, raised colonies. After 7-14 days, other colonies, much more glistening, slimy, and spreading, somewhat resembling a thin, watery starch paste, appear. These colonies, under the microscope, are found to consist of nearly spherical yeast cells, approximately 8μ in diameter, highly capsulated, and with a large globule of lipid nearly filling the cells.

Carefully touch the outer surface of such a slimy colony with a flattened inoculating needle, avoiding any sporangia of *Mucor* and related fungi, which are frequently present. The most difficult aspect of isolation is to purify the yeast by conventional streaking and yet avoid the rapidly spreading fungi of the Mucorales. Transfer the yeast from the needle to a drop of sterile water deposited on plates of the medium (M-37) and spread with a loop. As soon as colonies of yeast appear on any of the plates (2-3 days), transfer to another plate so that danger of becoming overgrown with molds is minimized.

Although spores can sometimes be observed in colonies on the original isolation plate after 2-3 weeks, the following sporulation medium yields good results: 1% inulin, 0.67% Yeast Nitrogen Base (Difco), 2% agar. Inoculate a fresh culture on slants of this medium. Sporulation occurs after 10-20 days. In heavily sporulating cultures the growth turns a light to dark brown, due to the amber colored spores. In *Lipomyces*, vegetative cells produce one or more large sac-like appendages or buds that initially have a granular appearance, in contrast to the homogeneous, refractive lipid globules in normal buds. The granular substance is gradually transformed into oval, amber colored, smooth surfaced ascospores, four to eight per ascus, occasionally even more. *Lipomyces* contains maximum fat when growing on a nitrogen-deficient medium. To confirm the lipid nature of the globules in *Lipomyces* cells, add 0.5 g of Sudan Black to 100 ml ethylene glycol, place on a shaker for one hour, and filter the solution. Place a cover slip on a *small* droplet of cell suspension. Deposit a droplet of dye on the edge of the cover slip from where it will be drawn under the cover slip by capillary forces and form a gradient concentration. The lipid globules stain deep blue to black.

For additional information, see Starkey, 1946; Lodder, 1970.

Isolation of Hansenula from Beetle Galleries

The two principal genera of pine bark beetles are *Dendroctonus* and *Ips*. Larvae and adults, as well as frass found in the galleries, are very high in yeast content. Presumably, the yeasts play a role in the nutrition of the larvae developing in the cambium. Certain species of *Hansenula* are particularly common in association with these beetles.

H. capsulata has been demonstrated in all of the major species of pine in California, and Wickerham has reported this yeast in a number of species of coniferous trees elsewhere in the United States and Canada. Frass or brood material of a recently killed specimen of any of these species of pine bark beetles is plated on a synthetic medium with glucose as the carbon source and nitrate as the nitrogen source (M-86). Colonies of

H. capsulata can be recognized by their slimy appearance, absence of hyphae or pseudohyphae at the periphery of older colonies, their small cells, and by the early formation of spores in the colonies of some strains.

A pine tree (any species) that is in the process of dying due to bark beetle attack can be recognized by the yellow or slightly brownish discoloration of the needles. When the trunk of such a tree is inspected, numerous holes are evident in the bark, through which pitch has exuded. A tree that has died some time ago is unsuitable, because the initial attack is followed by all sorts of secondary invaders that introduce many other yeasts and molds. With a hatchet, remove some of the bark and note the presence of larva and frass in the galleries. Bring the bark to the laboratory in a plastic bag. Scatter particles of insect frass on some plates and on each of several other plates deposit a larva from one of the galleries. Squash the larva with a needle or loop in a drop of sterile water and streak the larval parts over the surface of the plate. Incubate the plates at room temperature and watch for mucous colonies; these usually appear after 2-3 days.

Colonies of *H. capsulata* consist of very small spheroidal to ellipsoidal cells, rarely more than 1.5μ x 4.0μ. A second species of *Hansenula*, *H. holstii*, which also forms mucous colonies, has considerably larger cells (up to 4μ wide and 7μ long in young colonies) and, in older colonies, forms a periphery of true and pseudohyphae containing many elongate cells.

Select several colonies that appear to be *H. capsulata* and, after suspending some of the growth in a small amount of sterile water, restreak on 5% malt extract agar. Repeat the process on the same medium until a pure culture is obtained.

H. capsulata is a homothallic yeast that sporulates rapidly or slowly (depending on the strains) on 5% malt extract agar. Zygotes and spores sometimes appear within three days. The yeast is haploid and heterogamic conjugation (nearly always between mother cells and their buds) precedes spore formation. Asci contain a maximum of two spores, which are hat-shaped with a thin, often downward turning brim. Asci rupture soon after the spores are produced, and the released spores often remain together in pairs in a brim-to-brim position. *H. holstii*, on the other hand, is a heterothallic species and does not form ascospores in pure culture. The imperfect form of *H. holstii* has been described as *Candida silvicola*.

For additional information, see Wickerham, 1951; Shifrine and Phaff, 1956.

Isolation of Debaryomyces from Sausage or Bacon.

Species of *Debaryomyces* are generally tolerant to high concentrations of NaCl. They occur in ocean water and salt lakes and are associated with salted foods, especially meat products such as bacon and Wiener sausages. Most species ferment sugars only weakly. Species of *Debaryomyces* predominate in the slimy surface flora formed when bacon or sausage is stored at room temperature under relatively humid conditions. Other contaminating yeasts and bacteria can be eliminated by streaking the growth on a malt extract medium at pH 5.0 that has a high salt content.

Obtain several Wiener sausages and some slices of bacon, and store on pieces of waxed paper in a large Petri dish or similar container at room temperature and high humidity. After 3-7 days (depending on the freshness of the product) a slimy coating usually develops. This coating, upon examination, will be seen to consist largely of yeast cells. Prepare Petri dishes with malt extract agar (3% agar) adjusted to pH 4.8-5.0 and to which 15 g of NaCl per 100 ml has been added. With a needle suspend some of the slimy surface coating in 3 ml of sterile water in a test tube, and with a loop streak a drop of the suspension carefully on the surface of the medium. Exert very minimum pressure during streaking, since the high concentration of NaCl makes the agar less firm.

Incubate at room temperature for 5-7 days and pick a single colony for transfer and restreaking (after suspending part of the colony in sterile water as before) to an ordinary malt extract agar plate. It is also advisable to inspect the cells of the colony under the microscope, since *Debaryomyces* species generally consist of spheroidal cells that produce buds on all sides. If the second plate is not yet pure, transfer a single colony (after inspecting it microscopically) once more to a fresh plate of malt extract agar. When the culture is pure, i.e., if only one type of colony occurs on the plate, a single colony is picked with a loop and inoculated on a slant of Gorodkowa agar (M-49). Observe for the characteristic spores of *Debaryomyces*. (See also Mrak and Bonar, 1938a,b.)

Isolation of Saccharomycopsis from Rabbits

Saccharomycopsis guttulata is highly specific to the intestinal tract of both domestic rabbits and wild jackrabbits. It has also been reported in chinchillas and occasionally in guinea pigs. The pH of the stomach contents favors an active multiplication of the yeast, whereas in the remainder of the intestinal tract the cells are in a resting, nonbudding condition due to the higher pH, above 7.0, at which the yeast does not grow. Microscopic inspection of stomach contents or of fecal material should show the very large, ovoidal to elongate cells, often reaching 10μ x 20μ.

The yeast is most easily isolated from stomach contents but, because fecal material is more readily available, the procedure described below is based on fecal pellets. The organism is exacting in its growth requirements, requiring a balanced mixture of amino acids (as is to be found in yeast autolysate), growing only between 35 and 40°C, and, for growth on solid media, requiring a high concentration of CO_2 in the atmosphere. Additionally, it is very tolerant of low pH values of the medium.

● Obtain samples of fecal pellets of rabbits, preferably samples of soft feces, since the latter normally have a higher yeast content. Inspect the material microscopically for *S. guttulata* cells by suspending small samples in a drop of water on a slide. Use only material in which the yeast is present in significant numbers.

● Add one or two rabbit pellets or an equivalent amount of soft feces to 50 ml of isolation medium (M-50) in 125 ml Erlenmeyer flasks. Incubate for 2-3 days at 37°C, after which a whitish, granular sediment of yeast forms at the bottom.

- Although bacterial growth is inhibited by the low pH, occasionally other yeasts may compete with *S. guttulata* and form the predominant flora. Therefore, inspect the sediment under the microscope for the clusters and chains of the characteristic, large cells of *S. guttulata*. If the sediment appears satisfactory, transfer 1 ml of the growth with a sterile pipette to a second flask of the same medium and incubate 2-3 days at 37°C.
- After growth has developed, transfer 0.1 ml (or two drops) of the sediment to an agar plate (1% yeast autolysate, 1% proteose peptone, 2% glucose, pH 5.0) and spread with a sterile glass spreader. Place the Petri dishes in a desiccator with moist paper towels in the bottom to raise the humidity. Oxygen levels can be reduced by replacing the air in the desiccator with either gaseous CO_2 or nitrogen. If tanks of either type of gas are not available, a piece of dry ice about the size of a walnut can be allowed to sublime (provided a small crack is left to avoid pressure build-up).
- Inspect an isolated colony for purity. If bacteria have not been eliminated during the two previous steps, they will develop on the rich medium at pH 5.0. If the plate is found to be pure, the colony is transferred to a slant and to a liquid medium of the same composition as used above and incubated. Growth in liquid medium is always in the form of branched chains of cells, whereas on solid media the cells occur more typically in short chains, pairs and singly.

The characteristic ascospores can be obtained only at lower temperatures, best at 18°C, where vegetative growth is not possible. Prepare slants of a suitable sporulation medium (M-69). Tilt one of the cultures on liquid medium, prepared above, so that the growth collects at one edge and then remove 0.5 ml of the dense granular sediment with a pipette and spread it evenly on the surface of a slant of sporulation medium. Incubate the slants in a horizontal position at 18°C.

Observe for sporulation after 5-7 days by scraping part of the slant surface with a loop. Asci contain one to four ovoidal or spheroidal ascospores that lie free in the ascus. This arrangement is quite different from the tightly fitting spores in the asci of species of *Saccharomyces*. *Saccharomycopsis guttulata* cells are short-lived, but sporulating slants may be stored in a refrigerator for 6-12 months. Reinoculation of sporulating cells in liquid growth medium usually results in good growth after 5-10 days.

For additional information, see Shifrine and Phaff, 1958, 1959; Richle and Scholer, 1961.

Preparation of Nonrespiratory Vegetative Mutants

Among the many mutants of yeast that have been studied, the so-called nonrespiratory or respiration-deficient (RD) mutants are of special interest. Many kinds of yeast, including all species of *Saccharomyces*, possess both the respiratory pathway of metabolism (substrate combustion by oxygen consumption) and a second, the fermentative pathway (alcoholic fermentation). Ephrussi and his collaborators (Ephrussi, 1952) have shown that in a normal population of baker's yeast approximately 1% of the cells are abnormal in that they do not possess the ability to respire. When isolated, these abnormal cells represent a stable mutant type; after many transfers the respiration-deficiency is still present. Because the energy derived from fermentation is less extensive than that from respiration, the colonies produced by these respiration-deficient (RD) yeasts are smaller than those of normal types and have been called "petites."

It has been shown that certain dyes, in particular acriflavine (neutral acriflavine), strongly enhance the production of RD mutants in growing cultures of yeast. After several multiplications of yeast in the presence of suitable concentrations of dye, nearly the whole population will become respiration-deficient (Nagai, 1963).

Use a vigorous strain of *Saccharomyces cerevisiae* as the test organism, or suspend a small sample of compressed baker's yeast, obtained from a bakery or grocery store, in sterile water in a test tube and streak the suspension on a malt extract agar plate. Purify a single colony by one additional streaking.

Prepare a 100X strength solution of neutral acriflavine (Allied Chemical and Dye Corp.-National Biological Stains Dept.) by dissolving 15 mg acriflavine in 100 ml sterile water. The dye can be weighed on a piece of glassine powder paper.

Dissolve 12 mg Eosin Y and 17 mg of Trypan Blue (both Allied Chemical and Dye Corp.-National Analine Division) in 100 ml water and steam at 100°C for 50 min. This represents a dye solution in 10X concentration.

Prepare a medium containing yeast autolysate (Albimi) 2 g; glucose 20 g; agar 8 g; distilled water 360 ml. After autoclaving, when the medium has cooled to about 50-60°C, add 40 ml of dye solution as prepared above. Pour into Petri dishes.

Prepare two 250 ml Erlenmeyer flasks each containing 100 ml of medium (0.5% yeast autolysate and 5% glucose) and autoclave. After cooling, inoculate each of the flasks with a small loopful of baker's yeast from a slant. Add 1 ml of acriflavine solution to one of the flasks (thus giving a dye concentration of 1.5 mg per liter) and use the second flask as a control. Place at room temperature or in a 30°C incubator. Directly after inoculation, and at 6-12 hr intervals (for 24 to 48 hrs), streak a loopful of the two growing yeast cultures on two diagnostic plates. Cover the whole surface of the plate with the loop (bent under a 45° angle) so that well separated colonies are obtained. After growth (3-4 days) colonies of RD mutants show a brilliant purple sheen, whereas normal colonies are a soft greyish-violet. Note the progressive increase in the proportion of RD mutants in the culture containing acriflavine. Pick a single, small colony with a brilliant purple sheen and suspend in a small amount of sterile water. Restreak on a second dye plate and repeat until a pure culture is obtained. Then transfer the yeast to a slant of malt extract agar.

Effects on the Host

Symptoms of the parasitic phase vary widely, ranging from simple necrotic lesions to extremely elaborate galls, twig, flower and fruit deformations and witches' brooms. The major forms are discussed below.

Necrotic Leaf Spots, Blisters, and Leaf-curls The most common disease symptom caused by species of *Taphrina* is perhaps unthickened, necrotic leaf spots with no hypertrophy of host cells. Many species occurring on ferns, such as *T. polystichi*, and those on *Acer*, e.g., *T. sacchari*, as well as the very common *T. ulmi*, serve to illustrate this type of symptom. When hypertrophy becomes pronounced, it may cause the diseased tissue to bulge and produce a blister-like lesion. When such infected areas cover large portions or whole leaf blades, curling results. One of the commoner species that produces blister-like leaf lesions is *T. coerulescens*, while *T. deformans* on peaches is an excellent example of leaf-curl symptoms. There are many instances of symptoms that are transitional from leaf spots to blisters, to curls and, to a lesser extent, to galls.

Leaf Galls Some species (e.g., *T. filicina*) may, when extensive hypertrophy and hyperplasia occur, produce leaf galls. Such galls involve all the leaf tissue within an infected area. However, in several others (e.g., *T. californica*) leaf galls are epidermal in origin, although in older galls the mesophyll may become somewhat altered after the gall is fully developed. Leaf galls of epidermal origin are produced only by species that occur on ferns.

Enlarged Bracts The two species, *T. robinsoniana* and *T. occidentalis*, that occur on *Alnus* produce very elongate, tongue-like enlargements of the bracts of female catkins. They are often curved, saccate and contain vascular bundles that appear normal. The outer surface of infected bracts becomes covered with the ascogenous layer.

Plum-pockets Numerous species, including the very common *T. communis*, produce strikingly deformed fruits of plum known as "plum pockets." Enlargement of infected fruits becomes evident about 14 days after blooming. Eight to 10 days later, they reach full size, which may be 5 times that of uninfected fruits. The fruits, at first yellowish-green or even reddish, are later covered with a whitish bloom. They are hollow and relatively thin-walled. Species that produce pockets may also infect young stems and leaves and deform entire twigs. Similar symptoms also may be produced on cherries by several other species, including *T. farlowii* and *T. confusa*.

Witches' Brooms Witches' brooms may be produced by species of *Taphrina* in North America on *Alnus*, *Betula*, *Prunus*, and *Amelanchier*; none of these species of *Taphrina* occurs frequently in the U.S. Witches' brooms normally are formed by proliferation of foliage buds to form rather tightly-grouped clusters of twigs that curve upward. Individual twigs are usually elongated and thickened or may swell at the base. Frequently, young twigs die. Flower buds are absent, which gives the broom a dense green appearance. Leaves are usually somewhat thickened and may be slightly curled. Asci apparently occur only on the curled leaves when involved in the brooms.

Morphology of the Parasitic Phase

The mycelium in *Taphrina* is intercellular, subcuticular or located within the outer tangential walls of epidermal cells of the host. Intercellular mycelia of species like *T. populina* and *T. coerulescens* produce branches that grow outward between epidermal cells to form asci, whereas species like *T. deformans* produce mycelium that is at first intercellular but later develops a compact layer of subcuticular hyphae that develop asci. Still other species, including *T. betulae*, *T. carnea*, and

T. ulmi, have mycelia that develop in an entirely subcuticular fashion. Mycelium of several species, including *T. californica*, develops within the outer tangential wall of epidermal cells of hosts. Species with such mycelial habit do not occur commonly.

Intercellular mycelium is difficult to study, but it can be seen in free-hand or microtome sections stained by any standard procedure. The subcuticular mycelium and its development into a hymenium or ascogenous layer may be studied easily by clearing infected portions of host tissues and staining with KOH and phloxine (SR-13). Species like *T. ulmi*, *T. coerulescens*, and *T. deformans* that infect leaves are especially suited to study mycelial development.

Ascogenous Layer In species with intercellular mycelium only, hyphal branches emerge from between host epidermal cells to form asci. However, in species that develop subcuticular mycelium, these hyphae branch extensively to form a more or less continuous layer of short isodiametic cells that gradually separate from each other. An exception to the fragmentation of the ascogenous mycelium is found in *T. ulmi* where hyphae remain intact while asci are produced from most of the cells of the mycelium.

Ascus Development In species like *T. virginica*, *T. acerina*, and *T. acericola* asci are formed by rupturing the outer wall of ascogenous cells to allow the inner membrane to emerge and form the ascus wall. In *T. deformans*, *T. carnea*, *T. ulmi*, and *T. coerulescens* the ascogenous cell wall stretches to form the ascus.

In many species, including *T. polystichi*, *T. ulmi*, *T. robinsoniana*, *T. communis*, and *T. deformans*, a septum is formed across the basal portion of a developing ascus to cut off a stalk cell at the base and an ascus at the apex. In other species (e.g., *T. coerulescens*, *T. virginica*) no septum is formed. Instead, the entire ascogenous cell is converted into an ascus. Cross sections of preserved or dried diseased material stained and mounted in KOH and phloxine or lacto-phenol and cotton blue illustrate well the various types of asci.

Cytology The dikaryotic condition can best be demonstrated in cross sections of material with young ascogenous cells. Standard cytological stains are suitable. Such preparations will also usually show developing asci in which the dicaryons form the relatively large 2N fusion nucleus. In many species, including *T. deformans*, *T. carveri*, and *T. ulmi*, karyogamy is immediately followed by mitotic division of the diploid nucleus. The diploid nucleus that remains in the upper portion that will become the ascus soon undergoes meiosis. In species like *T. deformans*, *T. ulmi*, and *T. carveri*, eight haploid ascospore nuclei are produced; in *T. populi-salicis*, Kramer (1960) found that repeated nuclear division occurred, producing many ascospore nuclei. Still other species, such as *T. deformans*, become filled with many small spores as a result of ascospore budding.

The Saprophytic Phase

The species in question can be easily isolated by cutting portions of the host plant bearing infected areas and placing them in the lid of an inverted Petri dish containing an agar medium like potato-glucose. The ascospores will be shot upward and stick to the agar surface. Single clusters of spores can then be located with a low power microscope and removed with a micromanipulator, or isolated clusters may be marked and removed by cutting out a small portion of agar with a microscalpel. They result in single ascus cultures that develop rapidly on a variety of media (at 20-25°C).

If ascospores fail to reach the agar surface, then the portions of host tissue may be attached to the lid of the plate with an adhesive and the plate placed right side up. Asci then discharge spores downward and the spores settle on the agar surface. Since spores of a variety of other fungi, especially *Sporobolomyces* and other yeasts, may also fall on the agar surface, the plate should be examined after several hours and single clusters of ascospores removed. Contaminants may be held back by using such growth retardants as rose bengal or streptomycin to prevent bacterial development (Farrow, 1954).

All species of *Taphrina* develop budding yeast-like colonies that range from a salmon pink to pinkish yellow and have a characteristic dry yeast-like appearance on the colony surface.

For a monograph of the genus *Taphrina*, see Mix, 1949.

11 Plectomycetes

EUROTIALES

Gymnoascaceae

Because of their common occurrence as saprophytes on a variety of natural substrata and the ease with which they may be isolated and maintained in pure culture, members of this family are especially useful in the teaching laboratory. Under relatively simple cultural conditions ascocarps usually form in abundance. A single slide mount prepared from a young colony often provides all stages of development of sexual structures from the undifferentiated copulating initials through the formation of ascogenous hyphae, croziers and asci. Species of *Pseudoarachniotus* are especially useful in this regard, for they lack a well-defined peridium, and each ascogonium typically gives rise to a relatively small number of asci. In addition, the Gymnoascaceae are characterized by a variety of often bizarre ascocarps, and they form several distinctive kinds of asexual spores. The imperfect states of several species are the etiologic agents of dermatomycoses.

In all species of Gymnoascaceae where adequate studies have been made, the ascogenous hyphae arise from a well-defined, usually coiled ascogonium that is often closely associated with a presumed antheridial branch. In homothallic species the ascogonium and antheridium may arise from the same hypha or, as in the heterothallic species, from different hyphae of the vegetative mycelium. Several species of *Arthroderma* and *Nannizzia*, and *Ctenomyces serratus*, are heterothallic, but the majority of species comprising the family apparently are homothallic.

Various kinds of aleuriosporic or arthrosporic form genera are common to the Gymnoascaceae. A *Chrysosporium* state is associated with many species representing several genera of Gymnoascaceae; *Toxotrichum cancellatum* has an *Oidiodendron* state; species of *Nannizzia* are associated with species of *Microsporum*; one species of *Arthroderma*, *A. uncinatum*, is the perfect state of *Keratinomyces ajelloi*; and another species of the same genus, *A. quadrifidum*, is the perfect state of *Trichophyton terrestre*. *Microsporum*, *Keratinomyces*, and *Trichophyton* are dermatophytes. The imperfect states of several species of Gymnoascaceae are so similar morphologically that it is extremely difficult, if not impossible, to distinguish species of the former when they are not associated with their perfect states.

Occurrence and Collection In nature, these fungi are primarily inhabitants of soil, where they grow on a wide variety of organic materials, especially those containing cellulose or keratin. Such species as *Myxotrichum chartarum*, *M. deflexum*, and *M. ochraceum* are common on decomposing paper, cloth, and wood. Others, including species of *Arthroderma* and *Nannizzia* and *Ctenomyces serratus* frequent such substrata as rotting feathers, hair, horn and hoof tissues. Dung is a common substratum for many species of *Arachniotus*, *Auxarthron*, *Gymnoascus*, and *Pseudoarachniotus*.

During and following the rainy season one should look for Gymnoascaceae on any of the above materials that have been in prolonged contact with moist soil. Likely substrata also should be brought into the laboratory and maintained for a more or less extended period of time in moist chambers. The dung of such omnivorous rodents as rats and mice is often especially rich in species of Gymnoascaceae as well as other microfungi. Such animals feed on various insects, seeds, etc., in or on the soil and thus concentrate a wide variety of soil fungi in their feces. The dung of carnivores and herbivores is less productive but may nevertheless provide species of Gymnoascaceae. Lizard dung, too, is an excellent source. In the aird southwestern United States the dung of many kinds of small animals is readily available in the wild throughout most of the year.

Gymnoascaceae may be isolated from soil using direct plating or dilution techniques (Warcup, 1950; Kuehn *et al.*, 1961), but the culture of dung, wood, feathers, etc., is the most convenient means of obtaining a variety of species for class use. Soil baited with sterile hair, feathers, paper, etc., also is an excellent and simple means of obtaining certain of these fungi. Details of these two approaches are as follows:

- Moist chamber cultures, when maintained at room temperature (23-25°C), promote the emergence of ascocarps of any species of Gymnoascaceae present, usually within two weeks. These fruiting bodies may require an additional week or two to mature completely, and it is advisable to add water as required to keep the paper liner of the chamber moist. To do so, tip the dish, add a small amount of water near one edge, then rotate the dish so that the water flows around the edge. Allow the culture to dry thoroughly before attempting to isolate any Gymnoascaceae present. Gymnoascaceae, of course, will not be the only fungi present in such cultures, and they often may be completely overgrown by a variety of other organisms.
- To prepare baited soil cultures, fill a sterile Petri dish approximately one-half full with the soil to be examined and moisten it thoroughly with sterile distilled water. Bait the preparation with bits of sterile paper, cloth, feathers, or short lengths of hair--children's or horse mane or tail hair (Dawson and Gentles, 1961; Stockdale, 1963); the bait should be partially buried in the soil. Incubate at room temperature and observe regularly after about three days. Soil baited with hair or feathers is especially useful for obtaining species of *Keratinomyces*, *Microsporum*, and *Trichophyton*, several of which are known to have perfect states belonging to the Gymnoascaceae, i.e.,

species of *Arthroderma* and *Nannizzia*. Soil cultures, like moist chamber cultures, should be maintained for at least four weeks or longer in order to permit complete maturation of any Gymnoascaceae present. Appropriate measures should be taken to reduce the danger of introducing mites into the laboratory.

Isolation For routine isolation and cultivation of Gymnoascaceae, YpSs agar (M-70a) is one of the most useful. Nearly all nonkeratinophilic species grow and sporulate on this medium, and when it is supplemented with sterile hair several of the keratinophilic species also do very well. Simple wheat germ agar (M-33) has been found more satisfactory for several species.

When members of the Gymnoascaceae are growing in essentially pure stands, it is usually possible to transfer spores, especially those of the imperfect states, directly to agar media by a needle or fine tweezers. Usually, however, the ascocarps are formed in close proximity to, or are more or less overgrown by, other fungi. Direct transfer of whole ascocarps is therefore ineffective for rapid isolation, and a dry-spore isolation technique, as follows, is preferred.

- Swab an ordinary microscope slide with 95% alcohol, grasp the slide with tweezers, flood one side with alcohol and flame. Lay the slide with the sterile surface downward over the depression of Syracuse watch glass that also has been cleaned with alcohol.
- With the aid of a dissecting microscope select a mature, dry ascocarp of the fungus to be isolated. If it is overgrown with other fungi, carefully clear these away with needles or tweezers.
- Dip the finest tweezers available (preferably the kind used by watchmakers) in 95% alcohol, shake off the excess alcohol, pass quickly through the flame.
- Using the sterile tweezers, carefully remove the ascocarp from the substratum and transfer it to the cooled sterile surface of the microscope slide.
- Hold the ascocarp in place on the slide and by means of a sterile needle (a #0 insect pin inserted in one end of a match stick) shatter the ascocarp with sufficient force to scatter ascospores over about 1 cm^2 of the surface of the slide. The needle may be flamed

repeatedly and any large aggregates of ascospores transferred to other locations on the slide and shattered. In this way, often hundreds of small clusters of ascospores are spread over the surface of the slide, most of which are free of contaminating fungi.

- Using a magnification of about 20-25X, locate isolated clusters of ascospores and transfer them to agar. Flame the transfer needle (insect pin) and cool it in agar (this also wets the tip) prior to each inoculation. Four to five spot inoculations per plate of medium can be made. The spores of most species of Gymnoascaceae are light colored and show up best if a dark background is used on the stage of the microscope.
- After about 24 hours examine the isolation plates. Fungi showing rapid development within this period of time will not be Gymnoascaceae and should be removed. Most Gymnoascaceae are relatively slow-growing, but they should show definite signs of growth within three days. If obvious contaminants appear later, they can be eliminated easily by cutting them out and discarding them in a jar of 95% alcohol.
- As colonies develop, transfer portions of each to agar slants for preservation. Then allow the isolation cultures to mature completely--at least four weeks. Some species, like *Arthroderma curreyi*, lose sexual vigor on repeated subculture. However, in this species and most other Gymnoascaceae the primary isolation culture usually produces an abundance of ascocarps. Air dried ascospores of Gymnoascaceae are relatively long lived, and new cultures may be obtained readily from a specimen that has been dry for several years.

Maintenance and Storage Slant cultures of Gymnoascaceae on YpSs (M-70a), wheat germ glucose (M-33), or other suitable medium may be stored conveniently at 6°C in an ordinary refrigerator. Transfers need not be made oftener than once a year. Cultures also may be stored in a deep freeze (Kuehn *et al.*, 1961). Lyophil preservation, when available, is excellent for long-term storage.

Dried specimens for the herbarium or for class use may be prepared by spreading a thin layer of a liquid adhesive (white glue is excellent) over the inner surface of the lid of a suitable container such as a small pill box. Colonies growing on agar then are cut out and placed on the glue-covered surface and allowed to dry for several days. The glue will spread evenly beneath the moist agar, and when drying is complete, the specimen will be securely fastened in place. An appropriate label should be affixed to the upper surface of the lid of the container so that specimen and label cannot be accidentally separated.

Presentation Of the nearly 60 species of Gymnoascaceae currently recognized many are still imperfectly known; some have been collected only once. Many, however, are apparently common and widely distributed.

- *Gymnoascus reessii* (Orr *et al.*, 1963c, d) is extremely common and is the one most likely to be encountered on cultures of dung. An asexual state is unknown. This species grows and sporulates readily on YpSs agar. However, strains vary considerably in their ability to fruit in culture, and some do best at slightly reduced temperatures; hence cultures should be incubated at both room temperature and 18°C. Sexual structures should appear within six days. These consist of approximately equal clavate branchlets that twine symmetrically about one another (Benjamin, 1956, Pl. 7). Mature ascocarps should be present within three weeks.
- *Gymnoascus uncinatus* (Orr *et al.*, 1963c), known in most recent literature as *Myxotrichum uncinatum*, is also found commonly on dung. A *Chrysosporium* imperfect state is present in which both terminal and intercalary, finely asperulate spores are formed. *Gymnoascus uncinatus* grows readily on YpSs at room temperature. Usually the asexual state predominates in culture, and ascocarp formation may be limited or absent. Sexual initials are similar to those of *G. reessi*, but usually longer (Benjamin, 1956, Pl. 9)
- *Auxarthron brunneum* (Orr *et al.*, 1963a) is a common coprophilous species. The nearly spherical ascocarp is pale brown to dark brown or reddish brown. The peridial hyphae are anastomosed, smooth to asperulate, thick-walled and, like other Auxarthrons, characterized by enlarged septa or "knuckle joints." Short asperulate spines with slightly rounded or truncate apices as well as elongate appendages are present. The latter may

reach lengths of 1 mm and be straight, uncinate, or coiled apically; one to three knuckle joints are present near the base of each appendage. The ascospores usually are ovoid, 1.8-3.2μ x 2.2-4.8μ and minutely asperulate. A *Chrysosporium* imperfect state is present and both terminal and intercalary smooth spores are formed, usually not in great numbers. Ascocarps develop in abundance on both YpSs and wheat germ agars at room temperature. Initials may begin to appear in five days, and mature fruiting bodies should be present within three weeks. The aerial vegetative hyphae are nearly hyaline. Sexual structures consist of two apposed, broadly clavate initials, one much larger than the other. The smaller of the two gives rise to the ascogenous hyphae. Other species of *Auxarthron* commonly encountered on dung include: (a) *Auxarthron californiense* in which elongate, typically uncinate, appendages are present; and (b) *Auxarthron zuffianum*, lacking elongate appendages.

• *Myxotrichum deflexum* (Orr *et al.*, 1963b), like several other members of the genus, is usually found on decomposing cloth, paper, wood, feathers, etc., in contact with moist soil. It is not often found on dung. The ascocarps are very distinctive, brownish-black to black, bramble-like, with long radiating branches bearing straight or usually deflexed lateral branchlets. The ascospores, measuring 2.5-3μ x 4-5.5μ, are nearly colorless, elliptical-lenticular, and delicately striate with longitudinal furrows. Asexual spores are unknown. *Myxotrichum deflexum* is especially easy to maintain in the laboratory. It grows and sporulates readily on YpSs and wheat germ agar--best on the latter--at room temperature, and it is relatively stable on repeated transfer. Most strains produce a brilliant red pigment that diffuses into the agar substratum. Sexual initials form within a week, and ascocarps mature within three weeks. The ascogonium consists of a tightly wound coil that twines around the hypha from which it arises or, more rarely, a neighboring hypha. The coil may or may not envelop a globose or pyriform, short stalked, aleuriospore-like enlargement arising laterally from the hypha.

• *Myxotrichum chartarum* and *M. ochraceum* may be found in the same type of environment as *M. deflexum.* The former grows readily on YpSs agar at room temperature but usually does not produce many ascocarps, and those that do form mature slowly near the drying margin of the colony--especially in tube culture. The latter grows well on YpSs agar, but on this medium the colony remains rather pasty, and fruiting takes place slowly if at all. When the species is grown on wheat germ agar at 18°C, however, it quickly gives rise to a well developed aerial mycelium and forms an abundance of ascocarps within four weeks.

- *Pseudoarachniotus hyalinosporus* (Kuehn *et al.*, 1961) is found commonly on dung in California and northern Mexico, and has been collected in Iowa, England, and India. On dung it forms conspicuous yellowish aggregates of hyphae, often up to 1 mm in diameter, bearing large numbers of smallish clusters of asci. Each cluster represents the progeny of a single ascogonium. Ascospores are nearly hyaline to pale yellow, smooth, 2.2-2.3µ x 2.5-3.3µ. Asexual spores may be absent in some strains or represented by small numbers of arthrospores in others. The species grows readily on YpSs and wheat germ agars, and sexual structures begin to form within ten days at room temperature. The colony may consist of a nearly continuous aggregate of yellowish fruiting mycelium tinged bright green here and there. Mature ascospores should be present in about three weeks.

Preparation of Study Mounts Material for study may be prepared in any one of several ways, depending on use.

- The KOH-phloxine (SR-13) technique (Martin, 1952, p. 9) is one of the simplest and most useful methods for making temporary mounts. Treat a small portion of the material to be examined with the smallest amount of 70-95% alcohol that is needed to render it wettable; blot to remove excess alcohol. To the preparation now add a small drop of 2-3% aqueous KOH and follow this by a drop of 0.5% aqueous phloxine. In doing this, take care not to contaminate the stock solution of phloxine with KOH. A cover slip may be put in place immediately or the mixture of KOH-phloxine first removed by careful blotting and replaced with the KOH solution. After the cover slip is in place, carefully remove excess liquid and seal the edges of the cover slip with a 1:1 mixture of paraffin and beeswax (SR-18). The sealant is applied by means of a heated L-shaped wire easily fashioned from a large paper clip or ordinary shower curtain clip. Preparations may be used for several hours without appreciable drying.

- Two media are in common use for the preparation of semi-permanent mounts--Amann's (SR-1) and Lacto-fuchsin (SR-3). Treat the material (living or dry) to be examined with the smallest amount of alcohol needed to exclude air bubbles, blot to near dryness and add a small drop of the mounting fluid. It will often be necessary to arrange the material for better viewing, using small needles, before adding the cover slip. The edges of the cover slip then are sealed with nail polish or other suitable plastic material. Specimens mounted in water or KOH-phloxine may be infiltrated with either of the above media and made semi-permanent. Place a drop or two of the solution at one edge of the cover slip and draw it under by placing a bit of absorbent paper at the opposite edge. Allow the water to evaporate by permitting the slide to stand for several days. Add mounting solution as needed before sealing.
- Hoyer's medium (SR-7) is excellent for the rapid preparation of reasonably permanent mounts of the ascocarps of many Gymnoascaceae, especially those having pigmented peridial hyphae. The medium is useful for many other colored fungi as well. Its high refractive index renders it less satisfactory for hyaline structures, but this difficulty may be overcome in part by the incorporation of a dye such as acid fuchsin or cotton blue into the medium. Because it is an aqueous solution, fungi may be transferred to Hoyer's medium directly from water or alcohol. Dry material should be treated briefly with 70% alcohol and 2-3% KOH prior to transfer to the medium. Hoyer's hardens in time.

Eurotiaceae

Representative species of the Eurotiaceae that are easily cultivated include: *Eurotium repens, Emericella* *variecolor, Talaromyces vermiculatus, Thielavia sepedonium, Kernia spirotricha, Carpenteles javanicum, Sartorya fumigata,* and *Monascus purpureus.* (See p. 231 for an extended treatment of imperfect states in *Aspergillus* and *Penicillium.*)

Sources of Classroom Material Members of the Eurotiaceae can be isolated--but not dependably or regularly--from a variety of such natural substrata as soil, humus, stored grain, animal and rodent dung, moldy preserves or other products of high sugar concentration. Various techniques favor preferential isolation, such as Warcup's partial sterilization of the substrata in the autoclave, or use of media with high concentrations of sugar or salt. Generally, however, standard isolation techniques employing very weak, antibiotic-containing natural media, such as soil extract agar, are adequate for initial isolations. Strains are then transferred to Czapek's agar (M-89), malt extract agar (M-35-38), and malt extract agar + 1% yeast extract for optimum ascocarp development and identification purposes. Strains of the appropriate genera and species may also be obtained in pure culture from one of the major culture collections where named strains have been deposited.

Maintenance and Preservation Species of the Eurotiaceae do well on malt extract agar, malt extract agar plus 1% yeast extract, or potato glucose agar, as a rule, but *Eurotium* requires a high osmotic pressure medium like Czapek's agar in which 20% sucrose is substituted for the usual 3% sucrose.

When ascospores and conidia are mature, test tube slants can be stored in a refrigerator at temperatures of 5-7°C, and need be transferred into slants of fresh media only every 6-9 months. All members of the Eurotiaceae tested have been found suitable for lyophilization and, where equipment is readily available, preservation by this means may be preferred in order to minimize biochemical or morphological changes over a period of years. Slants also store well for several years when overlayered with mineral oil. Some workers maintain one set of cultures in a refrigerator for ready availability and a second, reserve set in lyophil or under oil to assure long-term availability.

Presentation Optimal culture media for maintenance of strains are also best for classroom study of the developing and mature stages. Colony pigmentation and variation of maturation are fairly striking on Czapek's agar, and this medium is recommended for use with each culture in addition to its optimal medium.

Ascocarp initials are particularly striking and in some cases are very useful in identification of species. They are usually best demonstrated in young cultures (approximately one week old) and stain nicely with a 1% aqueous solution of phloxine, staining more intensely than the surrounding mycelia. The variation in types of initials of *Carpenteles* and *Talaromyces* (genera with *Penicillium* conidial states) is beautifully illustrated by Emmons (1935).

Scheduling Preparations for Study

The length of time required for ascocarp maturation varies somewhat in the Eurotiales. It is suggested, therefore, that cultures be started at various time intervals prior to when they are desired. The following list summarizes lead-times and media needed for handling representative species exemplifying the Eurotiales.

Gymnoascaceae		
Byssochlamys nivea	2wk(M-89,35-38)	1wk(M-35-38)
Gymnoascus reessii	4wk(M-63a)	2wk(M-89,63a)
Shanorella spirotricha	4wk(M-63a)	2wk(M-89,63a)
Eurotiaceae		
Carpentales javanicum	4wk(M-35-38)	2wk(M-89,35-38)
Emericella variecolor	2wk(M-89,35-38)	1wk(M-35-38)
Eurotium repens	2wk(M-89,89*)	1wk(M-89*)
Kernia spirotricha	4wk(M-35-38)	2wk(M-89,35-38)
Monascus purpureus	2wk(M-89,63a)	1wk(M-63a)
Sartorya fumigata	2wk(M-89,35-38)	1wk(M-35-38)
Talaromyces vermiculatus	"(M-89,35-38)	1wk(M-35-38)
Thielavia sepedonium	2wk(M-89,35-38)	1wk(M-35-38)

M-89 = Czapek's agar with 20% sucrose

MICROASCALES--*(CERATOCYSTIS)*

Species of *Ceratocystis* are abundant in many areas and are rather easily obtained if one knows where to find them and how to isolate them. Some will develop perithecia within a week or less when grown on ordinary malt extract agar; a variety of conidial forms occur within the group.

All species are presumably disseminated by insects, although lumbering and sawmill operations may occasionally be a factor. The main sources for collecting are logs or fresh-sawn lumber. Logs that have been down six months or so and that are still moist will usually contain bark beetles, such as *Ips*, *Dendroctonus*, and many others. Cultures may be obtained by picking off perithecia from the beetle galleries with a transfer needle under a low-power dissecting microscope, by transferring ascospore masses from the tips of the perithecial necks, or by making isolations from the stained wood under the galleries and from stained lumber. Lumber that has been bulk-piled for a week or so after sawing will usually contain blue stain, and often perithecia on the surfaces. Old, dry logs and lumber are poor sources of cultures even though they may bear numerous perithecia on the surfaces.

Generally, it is difficult to keep species of *Ceratocystis* in fruiting condition over a period of time, as a result of which frequent reisolation is necessary. The most promising approach is to transfer ascospores from the tips of perithecia, working under a dissecting microscope. Cultures are best held in an ordinary refrigerator and transferred before drying out.

Some of the commoner species of the genus, with brief comentary on their characteristics, are as follows:

- *C. pluriannulata*--common in eastern forested areas on timber and logs, both hardwood and softwood. Conidia are exogenous; perithecia develop in about a week.
- *C. pilifera*--similar to above, but confined to softwoods in most areas of North America.
- *C. minuta*--to be found in galleries of beetles of genus *Ips* in conifer logs, both in eastern and in western U.S. Conidia are exogenous
- *C. multiannulata*--found on surface of green pine

lumber in the southeastern U.S. Conidia are exogenous; perithecia develop in about a week.

- *C. coerulescens*--the most commonly encountered fungus on stained Douglas fir lumber on West Coast; is easily isolated from stained wood at most mills. Endoconidia are present; should be grown at 70°F or below.
- *C. virescens*--most common on hardwoods that are damp and recently cut in the eastern U.S., especially on oak, magnolia, gum, beech, and maple in New England and the Southeast. Endoconidia are present; will grow at ordinary room temperatures of 75°F.
- *C. fimbriata*--causes black rot in sweet potatoes and can be recovered from cankers on sycamore and aspen, from wounds on fruit trees in California, and from coffee and other species in the tropics. Several conidial forms, including endoconidia, are formed.
- *C. minor*--found in relation to the galleries of *Dendroctonus* beetles on many conifer substrata in eastern and western U.S. (*D. brevicomis* in California, *D. pseudotsugae* in the Rocky Mountains and the Pacific Northwest) and on conifers unassociated with specific bark insects. This species is of special interest because of sclerotia that force apart layers of bark, making room for the tiny perithecia.

12 Pyrenomycetes

Despite the importance of ascocarp development in a modern classification of the Pyrenomycetes, the difficulties in presenting such data to the student are numerous and this phase is often neglected. But it is possible, through the selection of proper material, to demonstrate the major morphological features that are characteristic of the different taxa. Wherever possible, living material should be used, because many features of development that are readily seen in living material are difficult to demonstrate in dried specimens. The failure of most Pyrenomycetes to form ascocarps in culture, however, makes the use of some dried material necessary. All of the living material discussed below is grown on V-8 juice agar (M-30) or a modification of it.

For microscopic study of early stages of ascocarp development a 5% V-8 juice medium is used to reduce growth of aerial mycelium, which interferes with the observation of ascocarp initials. After Petri dishes are poured and the agar solidifies, place small squares (7-8 mm) of cellulose on the surface of the agar in a circle midway between the center and the edge of the plate. Use about six squares per dish. The preferred cellulose is Visking seamless cellulose tubing, obtained from scientific supply houses, which should be stored in a refrigerator to minimize the sticking together of the sides of the tubing. A small index card inserted in the tube and pushed through the tubing as pieces are cut off behind it will save many moments of anguish. Initially this can be done quickly and easily by dipping the end of the tubing in water, and then inserting the card. Water cannot be used routinely, however, since it causes the cellulose to wrinkle badly. Clear *unwaterproofed* cellophane can also be used, but is difficult to obtain in small quantities.

Sterility of the plates may be maintained by sterilizing the cellulose squares in a dry Petri dish when the medium is prepared, and then placing the squares on the medium with sterile forceps, being careful not to introduce contaminants. Or the propylene oxide method of sterilization can be used. In this case, after the squares have been placed on the agar, the plates are put in a dessicator or jar with a tightly fitting lid and propylene oxide added (1 ml per liter capacity of the container) and the plates left for 24-48 hours. After removal the plates are left overnight to permit any residual gas to dissipate. Since propylene oxide is flammable, it must be kept away from open flame and should be handled in a well ventilated room. With reasonable care, however, this is a safe, quick and easy method of sterilization, and it also avoids the biochemical changes in media that steam sterilization sometimes causes. Twigs, seeds, and herbaceous stems can also be sterilized by this method.

To prepare materials for class use, transfers can be made from stock cultures to fresh V-8 juice agar slants 10-14 days before they will be needed. Petri dishes should be inoculated with blocks of mycelium at the center of the plate. As the mycelium grows outward from the center, it soon covers the cellulose squares. If an imperfect stage is present, the formation of conidia can be studied at this time. A few days later ascocarp initials will be present, followed by young ascocarps. This technique works well for nonstromatic Pyrenomycetes, but prepared slides are necessary to demonstrate initials in stromatic species.

To examine the cellulose squares, carefully peel them off of the agar surface with forceps. Place a drop of water on the slide and place the cellulose on the water, being careful to avoid air bubbles. A few more drops of water are then placed on top of the material and a cover slip dropped in place. The cover slip should then be pressed gently to flatten the material and squeeze out air bubbles.

Mature ascocarps can be studied by placing several in a drop of water, adding a cover slip, and pressing so as to crack open the ascocarp and squeeze out the centrum tissues. If too much pressure is applied, tissues and ascospores will be crushed and rendered worthless. Young ascocarps will usually show centrum structures best, before they are distorted by the expanding asci. After observation in water, aqueous solutions of various stains may be used on both cellulose and whole mounts to bring out additional details. A few drops of a 0.5-1.0% solution of cotton blue or phloxine placed at the edge of the cover slip will usually stain the material adequately. Such rapidly growing tissues as young paraphyses or ascogenous cells having dense cytoplasm will stain more deeply than other tissues. Cotton blue in lactophenol works well and also permits the slides to be observed for several days. A few drops placed at the edge of the cover slip of a water mount will stain the material without collapsing it and will also allow one to observe the progress of the stain as it moves toward the center of the mount. In studies on the structure of the ascus tip, Waterman's blue-black ink is the best general stain. Cotton blue, aniline blue, and Janus green B may also give good results. The staining reaction varies with the stage of development of the ascus and the species being studied.

The staining of nuclei is more difficult but can be accomplished after a certain amount of persistence. Ascocarps must be carefully dissected and all pieces of the walls removed. The ascogenous system can then be further separated to give better staining. Dissection is done with the aid of a dissecting scope, using large plated sewing needles honed to a fine point. The material is placed in a drop of 60% acetic acid. To this is added a few drops of 1.0% orcein in 60% acetic acid, then a cover slip is placed on the material and the slide gently heated. The slide is then pressed in a paper towel to flatten the material. Additional stain may have to be added periodically until the slide is dark enough. Slides can be sealed with fingernail polish; two coats are usually necessary to complete the seal. Since chromosomes in different stages stain at different rates, periodic checks must be made to determine the progress of the staining. A certain amount of experience is needed to make good slides, but successful attempts are very rewarding.

Although a great deal of information can be obtained from the examination of fresh or dried material, prepared slides are very helpful in demonstrating certain stages in the development of many ascocarps. Such slides show the relative positions of various tissues, which cannot be done in squashes. A few such slides are available commercially, but these are usually of mature ascocarps and often do not show young material necessary to demonstrate critical stages of development. Thus, most such slides have to be made individually, by standard techniques.

Stock cultures can be maintained on V-8 agar slants at 6°C. Two slants of each culture should be kept so that an unused one is always available. Transfers of stock cultures should be made once a year; stainless steel or plastic caps help reduce drying in storage. If cotton plugs are used, a cap made of aluminum foil is desirable and will protect cultures from damage from dripping water in case of power or equipment failure. If very small tubes are used for storage, transfers must be made more frequently. Ascospores should always be transferred when possible, as this seems to aid in maintaining fruiting cultures.

Material on sticks or other substrates may be kept by air drying and storage in such suitable containers as boxes or envelopes. Paradichlorobenzene (moth balls) should be added to prevent insect damage. Dried material is often easier to section if first held in a moist chamber for a time before use. Fungi on leaves, such as the powdery mildews, are easier to store and handle if the leaves are pressed before drying.

Since most Pyrenomycetes discharge their ascospores forcibly, they can usually be isolated by inverting an agar plate over a few moist ascocarps. Several can be placed in the same dish. A circle marked on the Petri dish bottom with a wax pencil will aid in locating ascospores under the microscope. The spores will stick to the agar and can be seen under the low power of a microscope. Water agar is best for this as it is clear and the spores are easier to see, but any medium will do. Ascospores can be removed with a sterile flattened needle and transferred to any suitable medium. A number of isolations should be made, as contamination is not uncommon. Sordariaceous species are very good for this since their dark, relatively large ascospores can be easily located under a dissecting microscope. They occur on the dung of many kinds of herbivorous animals, and several kinds should be tried if possible. Rabbit dung is easy to work with, due to its small size, and can be incubated in a deep Petri dish. Dung may be stored and used effectively for many months.

The time intervals given below for the formation of mature ascocarps refer to cultures grown on a laboratory table at room temperature (ca. 25°C) with normal fluorescent lighting. Some variation from these times may be expected under different conditions. For this reason, two sets of class material should be prepared about three days apart to assure a ready supply of material of ascocarps of all ages. The use of a controlled temperature incubator will assure more uniform results but is by no means essential.

The fungi in this series of Ascomycetes are characterized by having a unitunicate ascus and a true perithecial wall enclosing the centrum. The ascocarp typically originates as a coiled hypha or hyphae whose cells become multinucleate and develop into the ascogonium and ascogenous system. As the coil develops, hyphae grow up to form an envelope around it to become the perithecial wall.

For additional information, see Miller, 1949; Luttrell, 1951a, 1955; von Arx and Müller, 1954; Munk, 1957; Müller and von Arx, 1962.

ERYSIPHALES

Source

Powdery mildews, being obligate parasites, grow only on living foliage, fruit, and stems. While they are of very common occurrence, attacking upwards of 1500 different hosts and often causing severe disease problems, they are not always readily available. They are world-wide in distribution but occur most abundantly in temperate regions. Many species are well adapted to conditions of low atmospheric humidity, and therefore their distribution and occurrence is not limited by moisture. Certain genera of higher plants appear to be more commonly affected by powdery mildews, but this depends upon host variety and race or strain of the mildew.

Each mildew genus appears to attack predominantly host plants of a similar type, often hosts within a particular family. *Phyllactinia* and *Microsphaera* are found primarily on deciduous trees and shrubs. *Podosphaera* is a common mildew of both pome and stone fruit trees. *Sphaerotheca* also attacks certain fruit trees but is common on roses and such small fruits as strawberries, brambles, currents, and gooseberries. *Sphaerotheca* and *Podosphaera*, while not limited to Rosaceae, are the mildew genera most commonly found on members of this large host family. *Uncinula* is a serious pest of grapes and is also widespread on certain tree species. The mildews occurring on grasses, and commonly on many herbaceous plants, are species of the genus *Erysiphe*. The three most prevalent species are *E. graminis*, *E. polygoni*, and *E. cichoracearum*. A certain degree of specificity with respect to higher plant family is observed with these mildew species. *E. graminis*, in its many biological forms, confines its attacks to members of the Gramineae. *E. polygoni* is the major powdery mildew on members of the Leguminosae, including some woody species, and the Cruciferae. *E. cichoracearum* is the most prevalent on members of the Compositae and Cucurbitaceae. However, recent observations suggest that some collections that have been ascribed to

E. cichoracearum may have been *Sphaerotheca fuliginea* (Kable and Ballantyne, 1963).

Most cultivated species of rose are susceptible to mildew, and the fungus is common wherever roses are grown. Powdery mildew is often prevalent on apple, grape, many wild grasses and cultivated cereals, cucumber and squash, many Compositae, sour cherry, pea, and lilac.

Various parts of the host may be attacked, depending to a large extent on the plant affected. On woody host species, the young leaves and shoots are most susceptible to infection. In severe cases the entire shoot apex, including both leaves and stems, may be completely covered with mildew. Such shoots are often stunted and deformed. In the case of herbaceous hosts, it is often the older leaves that are affected and where mildew development is most vigorous. Mildew infections may also be found on the fruit of many hosts.

While the asexual stage in the powdery mildew fungi is quite common, the sexual stage occurs much less frequently. Perithecia are most often found near the end of the growing season; however, the requirements for their production are not well defined. When attempting to collect perithecia, it is important to realize that with certain mildew-host combinations perithecia are extremely rare, whereas with others they occur rather frequently. Rose mildew is a good example of the first situation--while it is one of the most prevalent and widely distributed powdery mildews, perithecia are seldom found. Host plants on which perithecia are often produced include: apple, California Buckeye (in the West), grape, grasses, lilac, and many composites. If proper mating types of certain mildew species are available, perithecia can be produced in the greenhouse or in detached leaf culture (Schnathorst, 1959; Morrison, 1960).

Collection and Transportation

Powdery mildews must be collected from living plant material and the vegetative stage must be maintained on living hosts. These fungi can be collected and transported in a satisfactory manner by placing a sample of the infected host (leaves or shoots) in a plastic (polyethylene) bag, which is closed by means of a rubber band. If the samples are maintained at a moderately cool temperature, they can be kept for several days for transportation to the laboratory. No additional moisture need be added to the bag.

Maintenance in Greenhouse or Laboratory

If the fungus is to be maintained, it should be transferred as soon as possible to a suitable host plant growing in the greenhouse. This can usually be accomplished by transferring conidia directly to the host plant. If, however, conidia did not remain viable during transport to the laboratory, viable spores can often be obtained by placing the sample specimen under conditions of high humidity for 24-48 hours. Shoots or small branches can be maintained by placing the stem in water within a moist chamber, and individual leaves can be floated on water in a closed Petri dish. A camel's hair brush is very convenient for transferring conidia. After each transfer, the brush can be sterilized by rinsing thoroughly in 95% ethanol and then drying. Because of the unique water relations of the powdery mildews, it is not necessary to maintain the inoculated plants under conditions of high humidity in order to obtain infection. Even mildews whose conidia require very high humidity for germination on inert substrates (e.g., *Sphaerotheca pannosa* and *Podosphaera leucotricha*) will readily infect host plants under conditions of normal atmospheric humidity. However, it is advisable to have a relative humidity above 50%.

The preservation of fungal isolates for extended periods is often a problem, and the powdery mildews are no exception. These fungi can easily be maintained on suitable plants in a greenhouse, but this approach is not without difficulties. The conidia of powdery mildews are easily dislodged and moved about by air currents. Therefore, the infected plants will serve as a source of inoculum for infection of other plants in the greenhouse that are used for other purposes and on which mildew would be undesirable. In addition, it is difficult to maintain a specific isolate in this way because of the danger of contamination from wind-borne conidia from other sources. Mildew colonies can be maintained in detached leaf culture (Yarwood, 1946) but, depending upon the plant involved, the leaves deteriorate rather rapidly and repeated transfers are necessary. A simple method of detached leaf culture is to float leaves on water in Petri dishes that are maintained under artificial light in the laboratory. Several workers have found continuous illumination with light of 300-400 ft-c from fluorescent tubes to be quite satisfactory (Schnathorst, 1959; Morrison, 1960). Most leaves should last a month or more under these conditions. A solution of sugar, usually sucrose, at a concentration of 2-10%, is sometimes used to float leaves instead of water. In some cases this results in more vigorous mildew development and longer survival of the leaves. However, many mildews develop quite satisfactorily on leaves floated on water, and sugar may only contribute to contamination.

Storage of Perithecia

A second approach to the preservation of powdery mildew is to store the mature perithecia. Studies with *E. graminis* have demonstrated that perithecia can be maintained in a viable condition for as long as 13 years by dry storage at 10°C (Moseman and Powers, 1957). It would be expected that perithecia of all species could be maintained for a period of at least a year. A technique to obtain infection from perithecia was reported by Moseman and Powers (1957). When single ascospore infections were not required, perithecia that had been separated from the mycelial mat were placed on moist filter paper (one or more fruit bodies) and the paper was suspended at the top of a lamp chimney that enclosed seedlings of a susceptible host plant. The discharged ascospores fell on and infected the plants. When cultures from individual ascospores were desired, the spores were allowed to discharge on 2% water agar in a Petri dish. Individual ascospores were transferred with a fine needle to plants under a chimney. Infection was obtained from about 10% of the transfers.

Development on the Host Leaf

The use of detached leaves provides an excellent method for observation and study of the powdery mildews. When established in Petri dishes, these leaves can easily be handled in a laboratory. With a good dissecting microscope it is possible to follow spore germination, mycelial development, and sporulation. To observe these processes in greater detail, the parlodion strip technique is very useful. For this purpose a 2% solution of parlodion or collodion is prepared in a volume/volume mixture of diethyl ether and absolute ethanol. A magnetic stirrer is very helpful in dissolving the parlodion, which should first be cut into small pieces. A glass rod is used to apply and gently spread the solution on the leaf surface. It will dry in a few minutes to a transparent film in which the fungus is imbedded. The film is then stripped off the leaf and transferred with sharp-pointed forceps to a microscope slide, oriented with the mildew upward, a drop of stain placed on the film, and cover slip applied. Either cotton blue in lactophenol or acid fuchsin in lactic acid is a very satisfactory stain. This technique is particularly good for observing spore germination and appressorium formation.

A parlodion film on water can be used to obtain good spore germination in the absence of the host. A 2% solution of parlodion in amyl acetate is used, one drop placed on the surface of water in an 11 cm Petri dish. After most of the solvent has evaporated, a very thin film remains; conidia can then be dusted directly on the surface of the membrane. Water evaporates readily through the thin membrane, presumably providing a microenvironment somewhat similar to the surface of a host leaf. The Petri dish containing the membrane should not be covered, because water will collect on the surface and inhibit germination. After an appropriate period, a microscope slide can be slipped under the membrane, the unnecessary portion cut or torn away, the edges blotted to remove excess water, and stain applied, followed by a cover slip.

Morphology

The haustoria formed by powdery mildews can readily be observed, in most cases, in the epidermal cells of the host. With hosts from which the epidermis can easily be removed, such as barley and other grasses, a strip provides a good microscope mount. If a cross section is desired or if a satisfactory epidermal strip is difficult to obtain, e.g., with rose and peach, sectioning of fresh leaf material can readily be accomplished with either a hand microtome or a sliding microtome. For the latter, place the leaf segment in a slit cut in a block of fresh carrot, which is then secured in the microtome. Cut sections onto a film of water on the knife or razor blade by an oblique cutting motion, transfer them to water to float away carrot tissue, and then to a drop of stain on a microscope slide for observation. Another method for preparation of material for microscopic observation is to fix and clear the infected leaf with formalin-acetic acid-alcohol, followed by staining with cotton blue or acid fuchsin.

Perithecia can be viewed *in situ* with a dissecting microscope or mounted for observation under a compound microscope. Care should be taken when mounting perithecia because the characteristic appendages are easily broken. The contents of perithecia can be extruded by gently tapping the cover slip on the slide. This should be done with a sharp needle while observing under low power (10X) of a microscope to prevent unnecessary destruction. Pressure just sufficient to extrude the asci is all that is required. In many cases ascospores are not formed on the living host, and a period of maturation is required before they become delimited.

Moisture relations

The powdery mildews are unique among fungi in that conidia of many species are able to germinate under conditions of low atmospheric moisture. Considerable variation is exhibited within the group with respect to their tolerance of moisture stress. Free water is generally harmful to powdery mildew conidia; yet some species, e.g., *Podosphaera leucotricha* and *Sphaerotheca pannosa*, require conditions of high humidity for optimum germination (Berwith, 1936; Longreé, 1939; Weinhold, 1961). Other species, e.g., *Erysiphe cichoracearum* (Schnathorst, 1960), can germinate under conditions of moderate to high humidity. Conidia of several species, e.g., *Uncinula necator* (Delp, 1954), *Erysiphe polygoni* (Yarwood, 1936), and *Erysiphe graminis* (Yarwood, 1936; Cherewick, 1944), have been reported to germinate in an atmosphere free of water vapor.

A comparison of germination at various levels of relative humidity is valid only if temperature is held constant. A more meaningful expression of moisture stress is vapor pressure deficit (VPD), which is the difference between the observed vapor pressure and the maximum vapor pressure of water possible at the same temperature and atmospheric pressure.

The germination of powdery mildew conidia under various conditions of atmospheric moisture can, however, be demonstrated without critical humidity control. The moisture content of ambient air in most laboratories ranges from 30-60% relative humidity, which should be satisfactory for a moisture tolerance comparison of conidia produced by several species (*S. pannosa* with *E. polygoni*, *E. graminis* or *U. necator*). Conidia of other fungi might be included. In such an experiment the spores should be placed on an inert substrate such as a glass microscope slide, which is then exposed to the atmospheric conditions chosen. As a control, spores should be placed on glass slides that are held in a saturated atmosphere by suspending the slides over water in a closed Petri dish. Both systems should be incubated at the same temperature. Moisture condensation on the glass under conditions of high humidity may inhibit germination, and for this reason spores dusted on a parlodion film formed on water (Weinhold, 1961) provide a good additional control. The exact humidity at the surface of the membrane is unknown but must be very high because water evaporates rapidly through it.

An additional comparison can be made, under both of the moisture conditions, of germination on the surface of a host leaf. Leaves floated on water in a closed Petri dish provide a saturated atmosphere. Other leaves floated on water in an open dish can be exposed to the atmosphere. Moisture relations at the surface of a leaf are difficult to determine, but evidence presented by several workers indicates that the humidity at the surface more nearly approaches that of the surrounding atmosphere than of the intercellular spaces. Such a test also demonstrates the increased germination that is often observed on the surface of leaves, particularly with conidia of *S. pannosa*, which germinate poorly on glass slides. This beneficial effect is not clearly understood.

In studies of this type it is very desirable to have fresh conidia, obtained by gently brushing vigorous mildew colonies to remove all conidia at a specified time, generally 24 hours prior to setting up a test. After germination, spores on glass can be stained and observed; those on leaves can be removed using the parlodion strip technique.

If a more detailed demonstration of moisture effects is desired, a system of controlled atmospheric humidity is needed. Sulfuric acid is often used for humidity control, but because of difficulties in handling, saturated solutions of appropriate salts are more desirable. A very complete listing of useful salts is given by Wink and Sears (1950). Sucrose (Clayton, 1942) and glycerine (Scharpf, 1964) have also been used to regulate humidity. Stratification within the chamber may be a problem, and for this reason a mechanism for agitating the chamber is often employed. This problem can be overcome to a large extent by using a small container and suspending the spores close to the surface of the solution.

The conventional Warburg apparatus is very satisfactory for controlled humidity studies. The water bath will regulate temperature with a precision of ± 0.05°C and provide a shaking motion. The humidity regulating solution can be placed in the main compartment and small pieces of glass, on which spores are dusted, can be fastened to the side arm stopper. Vaspar (equal parts of paraffin and vaseline) is useful for attaching the glass pieces to the stopper. Methods for exposing detached leaves (Weinhold, 1961) and leaves attached to the plant (Delp, 1954) to conditions of various humidities have been reported. A system involving the passage of air through glycerine-water solution and through a chamber containing the sample has been described (Scharpf, 1964).

CHAETOMIALES

Species of *Chaetomium* can be readily isolated from most soils by placing strips of filter paper on the surface of moistened soil in a Petri dish. Within three weeks mature perithecia will have formed on the paper. The ascospores are exuded from the perithecium in a cirrhus and can be picked off with a sterile needle and streaked on agar plates. Most species grow rapidly and fruit abundantly in culture. Mature ascospores are formed in about four days on V-8 juice agar.

Of particular interest in this group are the evanescent asci and lateral paraphyses; i.e., paraphyses extending from the wall into the center of the perithecium. Some species, such as *Chaetomium globosum*, also have septate paraphyses among the asci. These break down early and can be seen only in young material. (See also Whiteside, 1957, 1961; Ames, 1963.)

SPHAERIALES

Although a number of fungi formerly included in the Sphaeriales are now placed in other orders, it still contains a diversity of forms. The morphological basis for the order is the presence of true paraphyses (i.e., basally attached) among the asci, but in only a relatively few of these fungi is the morphology well known. Even where it is known, there is disagreement as to the interpretation of data.

Sordariaceae

These are common saprobes occurring on dung and other plant materials. The large, dark brown or black perithecia are easy to work with and have distinct asci with large brown spores. The most common genera, *Sordaria, Podospora, Gelasinospora,* and *Neurospora,* are all easy to isolate in the laboratory. Species of the first three genera can be found on most kinds of dung, such as cow, horse, and rabbit. Pieces of dung should be placed in a moist chamber on paper toweling or filter paper. After a week or ten days, the black beaks or ostiolar necks can be observed protruding from the surface of the dung. The perithecia will be embedded in the surface of the dung and can be removed with the aid of a dissecting microscope. Isolations can be made by inverting an agar plate over a small piece of dung bearing mature perithecia. Ascospores will be shot off and will stick to the agar surface. They can be picked up individually or in groups with a sterile needle and transferred to nutrient agar. The percentage germination of many sordariaceous species is very low, but this can often be overcome by heating the ascospores at a high temperature (60-65°C) for 10-15 min, by placing the Petri dish bearing isolated ascospores in an oven.

Many species of *Sordaria, Podospora,* and *Gelasinospora* produce ascocarps readily in culture. The mycelium grows rapidly in culture. In *Sordaria fimicola,* young coils, the ascocarp initials, can be observed in three-day-old mycelium on cellulose squares. A light stain with cotton blue makes them stand out well. These fungi provide the best material for demonstrating the initial stages of perithecial formation. Young asci form after five days, and croziers can often be seen in whole mounts stained with cotton blue (but the nuclei are not differentiated). Material at this stage is good for cytological work. Ascospores mature after seven days. *Podospora* behaves much the same as does *Sordaria.*

In *Gelasinospora,* the formation of perithecia is somewhat slower than in *Sordaria.* Young coils appear on cellulose squares in three days; in four days trichogynes are clearly visible. Immature asci and ascospores are present after seven days. Paraphyses are particularly distinct at this time. By the 10th day ascospores reach maturity.

Species of *Neurospora,* as well as the other genera, are found in soil and can often be isolated from soil dilutions on agar plates. *Neurospora* ascospores must be heated to obtain good germination. (Compatible cultures of different species of *Neurospora* can be obtained from the Fungal Genetics Stock Center.) Cultures of *Neurospora* grow so rapidly and produce such an abundance of conidia that the use of a reduced nutrient medium greatly facilitates the study of perithecia. The formation of conidial chains can be studied on cellulose squares in two-day-old cultures. Protoperithecia are present after four days; these are best studied in unmated cultures to assure that fertilization has not taken place. Young asci will have appeared by the end of seven days, and mature asci and ascospores in 12-14 days.

In all of these genera the young asci will appear to be embedded in a pseudoparenchyma tissue. Paraphyses are frequently distinguishable in the upper regions of this tissue. Considerable confusion exists concerning the ontogeny of these fungi, despite the studies that have been made on them.

Neurospora is particularly valuable because of its importance in genetic and biochemical studies. It serves to demonstrate perithecial formation in a self-sterile, cross-fertile hermaphrodite. Ascospore color mutants can be obtained for both *Neurospora crassa* and *Sordaria fimicola*, giving visual evidence of the results of crossing-over with first and second division segregation.

Phyllachoraceae

Various species of *Phyllachora* are parasitic in grass leaves, often forming elongate streaks. Some of these mature in living leaves, others only after overwintering. *Glomerella cingulata* is a common saprophyte or wound parasite of many trees and shrubs. Several species of *Anisostomula* occur in leaves of *Quercus*, one in *Castanea*. Some are parasitic, forming and maturing in spots on living leaves; others mature in overwintered leaves. (See also Orton, 1944.)

Xylariaceae

While the ascospores of most Sordariaceae and Xylariaceae are similar, the complex structure of the apical apparatus in the Xylariaceae can be compared with the simpler structure in the Sordariaceae.

Unlike the Sordariaceae, species in the Xylariaceae rarely produce ascocarps in culture, so that dried material is usually needed for class work. *Xylaria* will often produce stromata in culture, but these remain sterile. Three of the most common genera are *Xylaria*, *Hypoxylon*, and *Rosellinia*. All grow on dead wood, often forming stromata over extensive areas of trees. Mature perithecia can be found during the summer and autumn. Nearly any fallen hardwood tree will contain some xylariaceous fungus before it begins to decay too badly. Paraphyses show up best in young material since they at times disintegrate as the asci mature. *Rosellinia* is particularly easy to study, since the perithecia are borne in a subiculum that generally covers only the lower portion of the perithecium. In *Xylaria* and *Hypoxylon* the perithecia are completely immersed in the stroma, with only the ostioles opening to the outside. Prepared slides of *Xylaria* show well the general characters of the family.

Diatrypaceae

These are stromatic fungi in which the stroma is composed of both fungus and host tissue. The asci in most species are long-stalked and contain allantoid ascospores. These are wood inhabiting forms, occurring on many species of dead wood in both dry and moist habitats. One of the more common species, *Diatrype stigma*, forms large black stromata on fallen logs. Another common species, *D. virescens*, forms small discrete greenish stromata on hardwood logs. These two species are readily seen in nature. Others, such as *Eutypella*, are buried in the wood with only the ostiolar necks protruding through the bark, and must be searched for. Still another genus, *Eutypa*, forms on decorticated wood, appearing as merely a black area on the wood. Examination of this black stroma with a hand lens will reveal the ostioles of the perithecia that are buried in the wood. *Eutypa spinosa* is very common in north temperate regions on fallen hardwood logs and branches. In searching for these fungi, it is frequently necessary to slice suspect twigs with a knife to reveal the fungus. The genus *Diatrypella* is interesting because of its polysporous asci. Few of the diatrypaceous fungi have been grown in culture, so the use of dried material is necessary.

For additional information see Wehmeyer, 1926; Cain, 1950; Moreau, 1953; Miller, 1961; Schrantz, 1961; Rogers and Berbee, 1964.

DIAPORTHALES

The fungi now included in the Xylariaceae, Diatrypaceae and the Diaporthaceae (Diaporthales) were formerly grouped together under the general heading of "stromatic Sphaeriales." In the Diatrypaceae and Diaporthaceae, in which the stroma consists of both host and fungus tissue, the relationship between the fungus and the host results in the development of different types of stromata. These types are an integral part of the natural biology of these fungi and are important in their taxonomy and classification. It will become apparent that there is no definite correlation between a stromal type and particular genera; some genera have different stromal types represented among the various species. *Diatrype stigma* and *D. virescens* both form a diatrypoid stroma. Many species of *Diaporthe*, *Valsa*, and *Eutypella* have a valsoid stroma, and *Eutypa* forms a eutypoid stroma. The latter is often evident in the wood only as a dark line delimiting the outer margin of the stroma, and thus is easily overlooked. In all of these stromal types it is necessary to make a clean vertical cut through the stroma to observe their differences. Most species of the large order Diaporthales form a distinct stroma composed of both host and fungus tissue, but in *Gnomonia* no such stroma is present. Various species of *Gnomonia* cause diseases of such plants as walnut, elm, cherry, and strawberry. The fungus can be collected in autumn on the leaves of the various hosts. Overwintering is usually necessary to obtain mature asci and ascospores. One species, *G. fructicola*, can be grown in culture and produces ascocarps, but special procedures are required to obtain perithecia in any number. Most species will have to be studied from dried or freshly collected material. *Lambro ulmea* on elm leaves and *Mamiania coryli* on *Corylus*, both conspicuous on living leaves, mature after overwintering. Species of *Gnomonia* are best found by examining leaves and petioles of many trees and shrubs in spring after overwintering. Beaks are usually elongate and slender. The asci in most species will float free in water mounts. Generally these bear a refractive ring at the apex, appearing in side view as a pair of cones.

The members of the Diaporthaceae are stromatic, forming in twigs and stems of both herbaceous and woody species. The most common genera are *Diaporthe* and *Valsa*. Both can be collected in late summer and autumn on dead wood. Usually only the ostiolar necks are visible to the naked eye, so that twigs must be split to reveal the perithecia. Diaporthaceous species do not produce ascocarps in culture, as a rule, although *Diaporthe phaseolorum* var. *sojae* can be isolated from diseased soybeans. It will form mature perithecia in about four weeks. Prepared slides of young material show the centrum well.

Melaconis spp. also are frequently encountered on branches of woody plants and *Endothia parasitica* may still be found on chestnut sprouts. A number of species in this order produce a distinct black stromatic line deep in wood beneath the grouped perithecia.

(See also Wehmeyer, 1934; Morgan-Jones, 1953.)

HYPOCREALES

This order contains two distinct groups, one represented by *Nectria* and *Hypocrea* (Nectriaceae and Hypocreaceae) and the other by *Melanospora* (Melanosporaceae). In the Nectriaceae and Hypocreaceae the centrum contains apical paraphyses; in the Melanosporaceae the centrum is pseudoparenchymatous.

Nectriaceae

The genus *Nectria* is the largest and most frequently encountered of the entire order. Species of *Nectria* are found on dead wood in relatively moist habitats during the summer and autumn. They are parasitic or saprophytic on branches of trees, readily recognized by the brightly colored, yellow to red perithecial walls. Some species are hypersaprophytic on larger stromatic Pyrenomycetes. They are widespread and can be found on both hardwood and coniferous substrates, but they are more common on the former. One species, *N. episphaeria*, grows on the stromata of other Ascomycetes, such as *Diatrype stigma* and similar fungi. Several species of *Nectria* cause cankers on hardwoods and can sometimes be isolated from them.

Most species of *Nectria* do not produce ascocarps well in culture. The most common species, *N. cinnabarina*, grows well and forms the conidial stage, but no perithecia form. *Nectria gliocladioides*, however, forms large stromata bearing perithecia in three weeks when grown on V-8 juice agar containing 1% glucose. This species is also interesting because it produces two conidial stages, a *Gliocladium* and a *Verticillium*. Both *N. cinnabarina* and *N. gliocladioides* are examples of stromatic species of *Nectria* in which the perithecia are borne on the surface of a broad stroma. Both show the apical paraphyses clearly in water mounts.

Nectria haematococca (*Hypomyces solani* f. *cucurbitae* Sn. & H.) is an example of a nonstromatic species. It fruits abundantly in culture, producing young ascocarps with croziers in 10-12 days. Mature ascospores are formed in 14-17 days. Apical paraphyses in this species must be observed in young material, since they swell and form a pseudoparenchymatous tissue at maturity. The fungus forms bright, fleshy perithecia, either red or white, on the surface of colonies grown on agar substrate. It is a *Fusarium* in the imperfect stage.

Nectria haematococca is heterothallic; an individual alone may be male, female, hermaphrodite, or neuter. Heterothallism is based on a single pair of incompatibility factors, which are not linked to factors governing sexuality. The male and female sex factors are linked, however, and this leads to the occurrence in a male X female cross of all four sexual types. Perithecial color is inherited independently of either sex or incompatibility.

This pathogen, causing root and stem rot of many species of the Cucurbitaceae, survives but a short time in the soil, so attempted recovery must begin with diseased host tissue. To isolate from diseased material, wash root and stem pieces in running tap water for an hour or longer; then surface-sterilize by immersing them in a 10% solution of Clorox or other commercial bleach for from 3-5 min, depending upon the size of the pieces. Dissect out small inner portions of tissue, approximately 3-5 mm in diameter, and place on acidified potato glucose agar. Acidify the agar at time of pouring by the addition of a drop or two of 25% acetic acid to each 20 ml of medium. Remove *Fusarium* sp. to fresh slants for further identification.

Hermaphrodite and female colonies on potato glucose agar are morphologically indistinguishable, bluish-green or brown, have brown stromata scattered on the surface, and produce abundant protoperithecia. This cultural type also sporulates profusely and is designated the C (conidial) type. Male and neuter colonies are identical morphologically, are white or occasionally light bluish-green, do not develop stromata, and produce no protoperithecia. This cultural type sporulates less abundantly than the C type and is designated the M (mycelial) type.

Maintain cultures on potato glucose agar slants in tubes with pH adjusted to 5.6. The 25 x 200 mm culture tube, although uneconomical of space, is convenient for manipulation of crosses. It also gives a large colony surface that can be easily examined for fruiting and pigmentation. Room temperature and diffuse daylight are suitable. Transfers must be made at least once every three months to insure continued viability. Transfers should be routinely accomplished with single spores, as mass transfers can lead to loss of a clone through overgrowth by mutants. This is particularly true of *N. haematococca* because of the inherent tendency for the hermaphrodites and females to mutate to male and neuter, respectively.

A number of spores from each individual culture should be isolated to insure retention of the original clone. In from 12-14 days, cultures started with single spores will be sexually mature. That is, they will have developed sufficient mycelium to produce numerous protoperithecia.

Spermatization is accomplished with this fungus by the transfer of microconidia or macroconidia to the surface of a receptive colony. Only hermaphrodite and female colonies can act as receptors in a cross, because male and neuter colonies form no protoperithecia. Either hermaphrodites or males can act as donors for spermatizing compatible receptors, but female conidia will not spermatize. Neuters are completely inactive sexually.

A mating pair, such as two compatible hermaphrodites, one carrying the factor for red perithecia and the other for white, can be selected as an example. Flood the surface of one of the cultures with 15-20 ml of sterile water and shake or scrape the surface of the colony with a transfer needle to insure a heavy spore suspension. Pour this spore suspension into the tube containing the other member of the pair, and again agitate or scrape. Then pour the mixed spore suspension back onto the first colony to produce a reciprocal spermatization. Once again, gently agitate, and then decant the water into the sink. Place the plugged tubes under conditions of diurnal light fluctuation.

Perithecia will mature in about 12 days. All on one colony will be red and on the other white. Ascospores from either type will yield colonies 50% of which will produce red perithecia, while the other 50% will produce white perithecia.

Because of the small size of the ascospores of this fungus, tetrad analysis is not feasible without the aid of a micromanipulator. However, analysis by population sample is fairly simple, involving the single spore technique. Under certain environmental conditions the ascospores are forcibly ejected from the perithecium, but in culture tubes they more often form a cirrus at the ostiole. If crosses have been made in culture tubes, it is more convenient at this stage to remove the slant of agar to a sterile Petri dish, as this facilitates picking up the ascospore masses. Using the transfer needle, remove the moist masses of ascospores to a sterile water blank and pour over the surface of the water agar for single-sporing. After spores have germinated, they should be outlined in the agar with three cuts of a chisel-edged needle. Before removing them from the dish, examine them under the 10X objective of a compound microscope to insure that only ascospores are being removed. At this magnification the characteristic two-celled ascospore can be easily distinguished from any macroconidia that may be present. This also eliminates the possibility of removing two spores accidentally lying together.

A female X male cross carried out as above will show development of perithecia on the female only. A population sample of ascospores will produce mature C type colonies and M type colonies in a one-to-one ratio. If test crosses are carried out, it is easy to demonstrate that four sex types are present.

Another fungus that produces mature ascocarps in seven days is *Neocosmospora vasinfecta*, which can be isolated from soils. Its development is the same as that of *Nectria haematococca*. *N. vasinfecta* produces handsome light-walled perithecia in culture that are especially effective for an introduction to perithecial forms because the student can mount whole perithecia and can see the asci as they develop in position inside the ascocarp.

A pathogenic species, *Gibberella zeae*, found on corn near the base of the stalks, has blue, warted perithecia.

Hypocreaceae

The Hypocreaceae have their perithecia embedded in a stroma with only the ostioles showing. The most common genus is *Hypocrea*, which grows on decaying wood in moist habitats. The lemon-yellow pulvinate stromata of *H. gelatinosa*, soon green over the surface, can be collected during the summer and autumn. Other species occur on old polypores and agarics. Fresh material shows the apical paraphyses well. *H. citrina* readily produces an effuse to pulvinate stroma with perithecia, asci, and ascospores when grown on cornmeal agar (M-5-7). Sections of dried material show the general arrangement of perithecia in the stroma that is characteristic of the family.

Hypomyces is a genus somewhat intermediate between these two families. The perithecia form in a subiculum, with the ostiolar necks protruding. It makes excellent material for demonstrating apical paraphyses since they show up well even in revived dried material. Some species are parasitic on agarics (*Lactarius* and *Russula*) and can be collected in late summer. *Hypomyces lactifluorum* is the most common species, distorting the fruiting bodies of fleshy species of *Lactarius* and turning them orange overall. The orange subiculum completely covers the underside of the pileus, with only small ridges remaining where the gills were. *H. luteovirens* is a green species on small *Russula* spp.; *H. hyalinus* on *Amanita rubescens*. Most species cannot be grown in culture, but one, *H. rosellus*, produces mature perithecia in three weeks on V-8 juice agar containing 1% glucose. Of particular interest in this genus is the large helically coiled ascocarp initial. Since these are embedded in the subiculum, they are best demonstrated in prepared slides.

In the examination of apical paraphyses care must be taken not to confuse them with the periphyses that line the ostiolar neck. Periphyses are short and more slender than apical paraphyses. The tips of periphyses also tend to be pointed while the tips of the apical paraphyses are blunt and rounded. Position is important: periphyses occur only in the ostiole while apical paraphyses are found in the centrum. While the young apical paraphyses are short they can be confused with periphyses but generally the latter will not have formed until later.

Melanosporaceae

The most common genus is *Melanospora*, which has a pseudoparenchymatous centrum and dark brown ascospores of a wide variety of shapes. Ascocarps are small, occurring on old herbaceous stalks, old polypores, etc. They can be isolated from soils, do not fruit well, and are difficult to maintain in pure culture, but they often grow well with another fungus, such as *Fusarium*. The nutritional relationship between the two fungi is unknown. Dried material can be used in class but will reveal little more than the shape of the ascocarp and the ascospores. Since the asci are deliquescent and the ascospores are exuded from the ostiole, isolations must be made by picking ascospores up with a sterile needle and streaking them on agar. *Phaeostoma sphaerophila* is hypersaprophytic on blackknot stromata (*Apiosporina morbosa*). It has two- or four-spored asci, oddly shaped one-celled ascospores. In the field it can be spotted readily--the protruding beaks provide a spiny, rough surface over stromata, very evident to the touch.

For additional information on Hypocreales, see Doguet, 1955; Booth, 1959; Hanlin, 1961b, 1963, 1965; Kowalski, 1965.

CLAVICIPITALES

The Clavicipitales are parasites on grasses, insects, and occasionally other fungi. The most common and best known species is the ergot fungus, *Claviceps purpurea*. It is abundant on wild grasses and is common on rye and wheat wherever they are grown. The perithecial stromata arise from sclerotia that form in the flower heads. Dried material and prepared slides show the characteristic filiform ascospores and perithecial shape, and the lateral paraphyses can often be seen in sections of young material.

It is possible to produce sclerotia on rye plants grown in the greenhouse by spraying flowering plants with a suspension of conidia. Sclerotia will form after about three months; then they must be cold-treated to obtain perithecial stromata. Sclerotia collected in late fall and buried in moist sand will produce perithecia in the laboratory. However, they require several months to develop. The conidial stage will grow in culture, but no perithecia form. It is relatively easy to produce perithecial stromata from *Claviceps purpurea* sclerotia by surface-sterilizing them and placing them on 1% agar slants at 10-15°C--development may take several months.

Another genus, *Cordyceps*, forms the perithecial stromata on insect bodies buried in soil. They are often found in moist woods but apparently occur at random, so that collection is uncertain but may be abundant in localized areas. The orange stromata of *C. militaris* are most commonly collected, on rotting logs in which the parasitized pupae are buried, during late spring and summer months. In the Northeast, we find *C. ophioglossoides* and *C. capitata* on *Elaphomyces* spp. during late summer and autumn. One species, *C. clavulata*, parasitizes scale insects and can often be collected on this host in late summer. Stromata are often abundant in an infected area.

A common parasite on grasses is *Epichloe typhina*, which is often found near ponds or wet areas. It will not grow well in culture, but dried material and sections show the essential characters well.

In the eastern United States, *Balansia epichloe* (= *Dothichloe limitata*) can be found on *Sporobolus poiretii* and, less commonly, on some species of *Calamagrostis* and *Panicum*. It is a systemic parasite and can be easily grown in the greenhouse on its grass host.

If material of several genera of this order is available, the variation in the shape of the stromata can be demonstrated. (See also Jenkins, 1934; Diehl, 1950; Mains, 1957, 1958; Doguet, 1960.)

CORONOPHORALES

These are rather infrequently collected and are not well known. The most common north temperate species is *Bertia moriformis*, which produces large, rough-walled ascostromata, often in large groups on rotting hardwoods. The asci are unitunicate, ascospores two-celled and elongate. (See Luc, 1952.)

CORYNELIALES

Many of the genera in the Coryneliaceae are tropical. Several species of *Caliciopsis* do occur in temperate regions, on conifers and on hardwoods. The fruiting bodies are long and flask-shaped; asci and ascospores are small.

In the Acrospermaceae, *Acrospermum compressum* is found infrequently on decaying leaves or grass culms. It has upright, elongate perithecia, long narrow asci, and filiform ascospores. (See Fitzpatrick, 1923; Brandriff, 1936.)

13 Discomycetes

REPRESENTATIVE SPECIES

Pyronema domesticum

This small, operculate Discomycete will produce mature apothecia in culture within a week and can be conveniently used to demonstrate mating structures and ascus development. The large sexual organs (clusters of club-shaped antheridia paired with globose ascogonia) of this species, their behavior during plasmogamy, and subsequent development of the asci and apothecia have been the subject of extensive research, as have those of *P. omphalodes* (= *P. confluens*). (See Harper, 1900; Claussen, 1912; Moore, 1963.) Developmental stages are readily found in cultures of the proper age.

Source of Materials Living material should be looked for in greenhouses where soil has been steam-sterilized for control of soil-borne diseases. The white to pink apothecia are often abundant on such steam-treated soil within one week after sterilization, but if soil was steamed more than two weeks previously, it is pointless to look for *Pyronema*, since more efficient secondary soil invaders will have crowded it out. Such apothecia will eject their ascospores to yield pure cultures when suspended over a solid nutrient medium. If one blows strongly at a sizeable mass of the apothecia, they will produce a visible puff or cloud of ascospores that can be caught on an opened dish.

Stock cultures can be maintained on test tube slants of Difco potato glucose agar (or any other convenient enriched medium).

Laboratory Study Begin cultures on days -6, -4, -3, and -2. Prepare the Petri dishes (disposable, divided type, 100 x 15 mm) by first marking the dish on the under surface of one sector with a wax marking pencil. Pour into this marked sector about 9 ml of medium B (M-95-b). After the poured agar solidifies, pour approximately 18 ml of medium A (M-95-b) into the remaining two sectors of the dish. Seed the culture by transferring a small piece of mycelium from the stock to the center of that sector of the dish that contains medium B. Incubate the cultures at room temperature in a lighted room; daylight is sufficient. Avoid light that is either extremely dim or intense.

Clusters of ascogonia and antheridia will begin to appear in about three days, and subsequent developmental stages will follow rapidly. Sex organs and young apothecia should be sought along the edge of the culture in those sectors containing medium A. The examination of the culture may be carried out either with a dissecting microscope or with the low power of the compound microscope. Young clusters of ascogonia and antheridia will resemble small clusters of bubbles. After locating sexual organs or young apothecia, lift the fungus with fine needles and place in a drop of cotton blue lactophenol on a microscope slide for further examination. If the material is young, it may be studied while intact, but if development of the sterile hyphae has begun, it will be necessary to pull the fungus apart with two fine needles before examination. Alternatively, very young stages may be more easily examined directly on the surface of the Petri dish culture by locating sexual organs as above, placing a drop of cotton blue lactophenol and a cover slip over them, and then examining with a compound microscope. Different stages of apothecial expansion may also be found by examining cultures of various ages with the dissecting microscope. The older cultures provide good material for study of ascus development.

If divided Petri dishes are not available, pour about 20 ml of medium B into ordinary disposable Petri dishes, using one dish for every four cultures needed. Pour 20 ml of medium A into the additional Petri dishes, using one dish for each culture to be established. After both media solidify, flame a scalpel and cut the contents of the dishes containing medium B into quadrants. Lift a single quadrant with the scalpel and transfer to a Petri dish containing medium A. Place the transferred quadrant so that the curved edge fits the side of the Petri dish. Inoculate each culture by placing a bit of mycelium in the center of the medium B quadrant.

Sclerotia Though true sclerotia are rare in operculate Discomycetes, *Pyronema domesticum* produces them abundantly on enriched media. They are tiny, globose, sterile (resting?) structures with a dark rind and a colorless medulla (Moore, 1962). They should be available in the stock cultures or can be prepared in quantity by growing Petri dish cultures on nearly any enriched medium (e.g., potato glucose agar or malt agar), and form within 10 days.

If living material of *Pyronema* is collected from fireplaces in the woods or campgrounds, one often encounters *P. omphalodes*, which will be satisfactory for demonstrating sexual organs, apothecia, and asci, but which does not produce sclerotia.

Ascobolus stercorarius

This coprophilous species grows on the dung of herbivorous animals. On this natural substrate, only the apothecia, 2-5 mm cup-shaped bodies, are readily visible. They are yellow when immature but later turn brown as the ascospores mature. A crop of asci matures daily, and about noon they simultaneously and violently eject their group of eight spores.

The species is especially suited to demonstrate sexual organs in a fungus. It is heterothallic (mating groups *A* and *a*) and forms sexual organs only after compatible strains (*A* and *a*) meet. The organs are large, distinctive structures that are easily discernible, particularly the ascogonium, under a 10X or 40X objective. Since the species is also hermaphroditic, two reciprocal interactions (*A* ♂ x *a* ♀ and *A* ♀ x *a* ♂) can be initiated and compared.

To obtain cultures, samples of dung (preferably cow dung) are placed in moist chambers; apothecia should appear within three weeks. Place a single, mature apothecium on the lid of an inverted Petri dish containing water agar (M-90). When the asci discharge, the spores will be ejected up onto the agar surface. They can be induced to germinate by heat-shock (60°C for 10 min) or by the NaOH method of Yu (1954). Mycelia of both mating types should be obtainable from a sample of about 10 germinated spores isolated into separate culture tubes. The mating types are determined by pairing the cultures (incubated at 22-27°C) in all combinations on fruiting medium (M-95-a). Apothecia will form only in compatible combinations.

The most vexing problem encountered with this species is that stocks kept on agar slants invariably lose the ability to produce ascogonia. This problem has been circumvented, however, by maintaining oidia under conditions where they do not germinate, but do remain viable. This is achieved in the following way: Aerial mycelium is scraped from a 7-10-day-old culture, immersed in 2 ml of 0.04 M phosphate buffer (pH 6.7) in a test tube, and stored in a refrigerator. When cultures are needed, a 0.1 ml sample is removed and plated onto fruiting medium. The resultant colonies invariably are able to form ascogonia.

Fertilizing Element Two distinct types of fertilizing elements are effective in mating experiments with this fungus.

- Nongerminated oidia--All mycelia produce large numbers of oidia (asexual spores) on the aerial hyphae. To obtain a suspension of oidia, scrape the aerial mycelium from a 7-14-day-old colony, suspend in a test tube of distilled water, and, if desired, filter through cotton to remove hyphal fragments. With a small bacterial loop or Pasteur pipette place a drop of the suspension (or a 1:10 dilution of it) onto the surface of a square of water agar (5 mm^2). *A* oidia should be placed on a square cut from a plate containing 6 ml of the water agar, *a* oidia on one from a 3 ml layer.

- Severed hyphal tips--Cut single hyphal tips from day-old cultures growing on water agar. The square of agar bearing each tip should be at least 5 mm^2.

In contact with an appropriate receptive mycelium, these fertilizing elements will differentiate as antheridia. In the first instance, differentiation is physiological; in the second, it is both morphological and physiological.

Receptive Mycelium Cut small blocks of agar from young cultures that are 2-7 days old and transfer these to the center of several plates of fruiting medium. After incubation for four days, the newly developed mycelium is receptive, i.e., will form ascogonia in the presence of an appropriate fertilizing element.

Mating Actual "mating" is performed by transferring a block of agar bearing a fertilizing element to each of several (four is suggested) "windows" in the plates of fruiting medium containing receptive mycelia. (Make "windows" by scraping away any aerial mycelium to insure intimate contact between the two agar surfaces.) Since the sphere of influence of each interaction never extends beyond the block of agar, four different combinations can be made in each plate. A typical procedure is to place a block bearing *A* oidia at window #1, one with *A* severed hyphae at #2, *a* oidia at #3, and *a* severed hyphae at #4. Thus, with just two plates, one bearing a mycelium of *a* mating type and the other of *A* type, four compatible (*A* x *a*) and four incompatible (*a* x *a* and *A* x *A*) combinations can be set up.

If the surface of each block is scanned periodically, the following phenomena will be seen. In all compatible combinations, antheridia will differentiate on the severed-hyphae fertilizing elements within 3-6 hours. In the case of severed hyphae, this differentiation is manifested morphologically by the transformation of the primary tip and of the side branches into elongated, slightly broadened antheridia. As for nongerminated oidia, differentiation into antheridia is inferred by the subsequent reaction of the underlying mycelium. In response either to oidia or to differentiated severed hyphae the receptive mycelium produces numerous thick, closely branched hyphae that grow up onto the agar surface and on which ascogonia will appear within eight hours. With luck, plasmogamy, i.e., fusion between an antheridium and the narrow apical part of the ascogonium, the trichogyne, may be observed. Soon thereafter (and sometimes even before), nearby hyphae of the mycelium will proliferate around the fused organs and quickly form the anlage of the apothecium.

In all incompatible combinations, none of these structures will appear.

The two reciprocal, compatible interactions will show consistent differences in the distribution of the ascogonia, particularly on the blocks bearing numerous oidia. In the combination *A* oidia + *a* mycelium, the ascogonia will form only in a narrow circle just outside the area containing the oidia. In the reciprocal combination, *a* oidia + *A* mycelium, the ascogonia will form within this area.

For additional information, see Bistis, 1956, 1957; Bistis and Raper, 1963.

Lambertella

Certain cultures of this genus will produce mature apothecia in culture if grown in oatmeal agar (M-15) in 60 mm Petri dishes, half full. Inoculations should be made 35 days before they will be needed and the cultures held at 17°C in light of about 400 ft-c so timed as to give 16 hours on, 8 hours off. Cultures held continuously in the dark will not fruit. (See also Dennis, 1968.)

Peziza quelepidotia

Cultures of this species will produce mature apothecia on Jiffy-7 Pellet infusion agar (M-95c) within two weeks when inoculated with ascospores or hymenial pieces. Cultures should be incubated under continuous fluorescent illumination at room temperature (ca. 25°C). At maturity, the apothecia are stipitate-pileate and generally 1-2 cm in diameter. The violent discharge of ascospores is quite dramatic, showing a visible cloud of spores. Air-dried apothecia stored at -20°C for six months have produced mycelia with mature apothecia within two weeks when placed on the above medium.

PRODUCTION OF APOTHECIA IN CULTURE

Several species of *Ascodesmis* readily form tiny apothecia (scarcely more than clusters of operculate asci intermingled with a few paraphyses) on potato glucose agar at room temperature. Mature asci containing large, brown, reticulate ascospores are formed within six days. *Anthracobia* also forms small apothecia in about five days on cornmeal agar at room temperature. In this latter fungus the ascospores are hyaline and contain two oil guttules.

In addition to producing apothecia clothed with hairs, and hyaline biguttulate ascospores, cultures of *Trichophaea abundans* (often called *Lachnea,* or *Patella*) will produce an abundant conidial state similar to *Botrytis* on rich media such as potato glucose or glucose-yeast agar. It has proven one of the more dependable species of Discomycetes for production of apothecia in pure culture and is very useful for showing all stages in the development of apothecia, asci, and ascospores. This species is apparently homothallic.

A suggested medium for production of apothecia of *T. abundans* is glucose 3 g, yeast extract 1 g, one 1-A-Day multiple vitamin tablet, agar 20 g, distilled water 1 liter. No adjustment of pH is necessary. Many variations on this medium may be satisfactory, but the medium must be dilute; higher concentrations of nutrients result in abundant conidia and fewer apothecia. Vitamins (thiamine in particular) and the microelements may be added separately but, for some reason not fully understood, the multiple vitamin tablet has given larger, more typical apothecia.

Light favors normal production of apothecia. Incubation should be between 20° and 25°C, under continuous artificial light (about 50-100 ft-c) or in alternate light and darkness. Placing the cultures near a laboratory window (but not in direct sunlight) should be satisfactory. Continuous light in an incubator has given consistently good results.

Abundant conidia of the *Botrytis* state are formed within 2-3 days. Apothecia with mature asci may be present as early as 7-10 days, depending somewhat on the temperature. Ascogenous hyphae and asci in all stages of development are evident at this time. For microscopic observation, entire apothecia may be placed on a glass slide and crushed gently. Apothecia increase in number and size during the second week, reaching a maximum of about 25-100 per plate and 1-2 mm in diameter. Mature asci may be induced to discharge ascospores, and the operculum is usually visible. Ascospores and conidia are readily distinguishable. This species appears to be stable in culture, and it continues to produce apothecia with no loss in vigor two years after isolation.

DE BARY BUBBLES IN ASCOSPORES

Ascodesmis spp., *Anthracobia* sp., and *Trichophaea abundans* all readily produce the gas-inclusions called DeBary bubbles (Dodge, 1957) when mounts of mature spores are placed in a strong mountant (e.g., lactic acid, glycerine, Melzer's reagent, 50% sucrose or dextrose). Students may be intrigued to watch these form (they "pop" into view) by mounting spores first in water and then replacing it by drawing through a strong mountant with filter paper. The DeBary bubbles increase in size (and number) with time, and may be made to decrease in size and finally disappear if water is again drawn through to replace the mountant. *Pyronema* ascospores will not produce DeBary bubbles, however. Students should also note that in those species where DeBary bubbles do form, ascospores that collapse in the strong mountant rarely if ever form the gas inclusions, and that young ascospores fail to form them.

14 Laboulbeniales

Most of the nearly 1500 described species of Laboulbeniales (in about 113 genera) occur on true insects, but several species of *Rickia* and *Dimeromyces*, as well as two species of *Laboulbenia*, are found on mites. A single species, *Troglomyces manfredii*, has been described on a milliped, *Troglojulus mirus*. Most of the species occurring on Hexapoda parasitize members of the order Coleoptera, but species also are known on representatives of eight other orders of true insects. Only a few species have been found on several of these major groups, however.

All Laboulbeniales are relatively minute and inconspicuous. Few are longer than 1 mm. Some species, such as *Zodiomyces vorticellarius*, are characterized by a relatively large multicellular thallus, or receptacle, bearing numerous reproductive structures and accessory sterile, uniseriate, cellular appendages. In most species, however, the thallus is reduced to but a few cells--two in many genera--giving rise to a single perithecium and one of several appendages that may or may not bear antheridia. Both monoecious and dioecious species are known.

The best readily available treatment of the Laboulbeniales is that by Gaümann and Dodge (1928). The illustrated monographs of Thaxter (1896, 1908, 1924, 1926, 1931) are essential for any serious attempt to determine most genera and species. Useful keys are not available.

The order includes many common and widely distributed species, and it often is possible to obtain representatives of several genera from insects living in a single ecological niche.

For additional information see also Faull, 1912; Spegazzini, 1917; Colla, 1934; Smith, 1946; Collart, 1947; Benjamin and Shanor, 1950, 1952; Richards and Smith, 1955a, b, c.

COLLECTING INSECTS

Equipment Needed

Likely hosts for Laboulbeniales are found in a great many different environments. A medium-sized fishing tackle box is ideal for transporting much of the simple gear that will be needed: (1) tweezers for handling both living and dead insects; (2) a hunting knife and three-pronged garden weeder for stripping bark, opening rotten logs and stumps, digging in leaf mold; (3) a quantity of assorted vials provided, preferably, with rubber or neoprene stoppers; (4) a small funnel of the kind used for filling baby bottles; (5) a hand lens; (6) a bottle of preservative; (7) an aspirator and killing bottle; and (8) labels, pencils, etc.

In addition to the above items, several pieces of more specialized equipment are essential:

- A modified butterfly net, in which the bag is constructed of unbleached muslin, for trapping Diptera and other insects on the wing.
- A wire-mesh food strainer, 6-8 inches in diameter, and a tea strainer for capturing aquatic insects.
- An aspirator for capturing rapidly moving terrestrial insects. This is one of the most useful pieces of equipment for collecting many kinds of insects. To construct it one needs a 2 ounce wide-mouth glass bottle; a two-hole rubber stopper with openings to permit the insertion of 1/4 inch copper tubing; a 6 inch and a 4 inch length of 1/4 inch copper tubing; a 24 inch length of rubber tubing to fit over the copper tubing; a small

piece of fine-mesh cloth; a short length of plastic tape or string. Construct as follows:

(1) Ream out both ends of the pieces of copper tubing. Make a slight median bend in the 6 inch length and a somewhat greater bend in the 4 inch length; insert both into the rubber stopper. The longer piece should project about 1-1/2 inches into the glass bottle; the shorter piece should project only 3/4 inch.

(2) The longer piece of copper tubing is the one through which captured insects will pass and is left open at both ends.

(3) Slip one end of the rubber tubing over the outer end of the short length of copper tubing.

(4) Carefully wrap the inner end of the short length of copper tubing with at least two thicknesses of the porous cloth and fix securely in place with the tape or string.

● Prepare several killing bottles using the same size bottle as for the aspirator. Each should be provided with a rubber stopper into the lower end of which has been inserted a short length of glass tubing containing sodium or potassium cyanide held in place by a small wad of cotton. A larger killing bottle, with a larger cork, should be available for large insects that are captured by other means.

● A sifter is very useful for collecting active insects that inhabit ground litter, flood debris, heaps of dead vegetation, the contents of hollow stumps, etc. It is simply a heavy duty sieve having 8, 10, or 12 meshes to the inch. The length and width of the sifter should be such that it fits into a flat-bottomed white-enameled tray about 3 x 10 x 16 inches.

● A Berlese funnel is an excellent means of collecting many of the smaller and slower-moving insects to be found in the same habitats as those for which the sifter is used. The Berlese funnel consists of a rather large metal funnel, at least 10-12 inches in diameter at the top, with a 1/4-1/8 inch mesh screen placed about one-third of the way down the funnel. The screen may be either soldered in place or supported by several angular metal brackets soldered to the side of the funnel.

Capturing Insects

To capture an insect with the aspirator, put the end of the rubber tubing in your mouth, aim the intake tube at the insect, and when you are about to touch the insect, suck vigorously. Insects collected by means of the aspirator may be killed simply by interchanging the cork of the aspirator for that of the killing bottle.

In using the sifter, place material such as leaf mold in the sifter and shake it over the tray. As insects fall into the tray, they are captured with the aspirator. Sturdy oil cloth may be substituted for the tray but is not as satisfactory.

The most effective use of the Berlese funnel is to suspend it in a large ring stand or other support with the small end inserted into a container of such preservative as 70% alcohol (it is best to add the preservative after the funnel has been loaded with the material to be examined). Leaf mold or other material is placed on the screen and spread toward the sides of the funnel so that a central opening extends downward to the screen. A cloth cover to prevent escape of insects is then stretched over the top of the funnel and held in place by means of a large rubber band. Finally, heat is applied to the material on the screen by placing an electric light with a suitable reflector directly over the cloth cover. As the material dries, the insects migrate downward and eventually fall into the preservative. Drying should not proceed so rapidly, nor should the heat be so intense, that the insects are killed before they have a chance to work their way to the bottom.

One or more Berlese funnels may be maintained in the laboratory. Samples are easily transported from the wild in coffee cans provided with plastic lids.

Preservation of Insects

Representatives of all of the groups of insects that are commonly infested with Laboulbeniales may be preserved in 70% alcohol. Insects stored in liquid are much easier to manipulate when they are being examined for parasites than are dry insects. Insects that have been killed in the killing bottle may be readily transferred to a vial or bottle of alcohol by means of a small funnel held directly over the opening of the container. After freshly collected insects have been preserved in 70% alcohol for a day or two, the fluid should be changed.

For an excellent discussion of the collection and preservation of insects from the standpoint of the entomologist, see Ross, 1962.

COLLECTING IN VARIOUS HABITATS

Aquatic Environments

Beetles belonging to the families Carabidae and Staphylinidae are parasitized by a variety of species and genera of Laboulbeniales, and these insects are especially common in damp to very wet situations. Representatives of these, as well as other families of insects that serve as hosts for these fungi, will be found near water: (1) crawling on mud and sand; (2) burrowing in mud and sand; (3) under stones, logs, wood fragments, and piles of flood debris; (4) in moss on exposed stones, logs, etc.; (5) under dead animals and plants.

Crawling insects are collected directly with the aspirator, or debris can be sifted over the trapping pan and insects collected as they fall through. Sand, mud, or debris also may be immersed near shore; insects will float to the surface of the water and are easily captured with a tea strainer. Large beetles may be caught with tweezers and placed directly in preservative or a killing bottle.

Aquatic beetles belonging to the families Gyrinidae (swimming on the surface), Hydrophilidae, Dytiscidae, and Haliplidae (crawling and swimming under water) are most easily collected by means of a wire-mesh food strainer or a tea strainer. Many are active swimmers and thus are very conspicuous and readily sighted. Species of Hydrophilidae and Haliplidae are somewhat sedentary and spend most of their time crawling on submerged vegetation in quiet shallow water. However, if one violently agitates the water, mud, sand, algae, or debris in which and upon which they are living, these insects often float to the surface in great numbers. Transfer the beetles directly to 70% alcohol using fine tweezers or gently tap the inverted strainer over a wide-mouth jar containing preservative.

Flies are often extremely numerous around mud flats and shallow pools of stagnant water. These insects, which often bear species of *Stigmatomyces*, are easily collected by sweeping over the surface with the modified butterfly net. Flies and other insects collected in this manner are transferred to a killing bottle and then preserved in alcohol.

Leaf Mold and Litter

Sift or process in a Berlese funnel such materials as damp leaves, leaf mold, woody debris under logs and in hollows in trees and stumps. Also collect from decaying grass piles, hay stacks, accumulations of debris in gardens, cultivated fields, and along cut-over road sides.

Foliage and Flowers

Beat or shake the branches of trees, shrubs, and herbs over the collecting pan or a large cloth spread on the ground and collect the insects with an aspirator.

Buildings

The genus *Herpomyces* is found only on cockroaches (Blattidae) and several species parasitize common household pests. The fungi usually develop on the antennae.

MATERIALS FOR HANDLING INSECTS

Tools and Equipment

- Tweezers--preferably high quality watchmaker's forceps kept sharply pointed by means of a fine-grained grinding stone.
- Depression slides. Two kinds are needed: (1) Large concavity slides (Maximov Tissue Culture) having a depression about 35 mm in diameter and 5 mm deep for manipulating insects during removal of parasites; (2) ordinary depression slides for temporary storage of parasites after their removal from insects.
- Microscope slides and 18 mm circular cover slips (#0 or #1).
- High quality minuten insect pins mounted in match sticks to use in removing parasites from insects. These needles are kept sharply pointed.
- Flat-bottom porcelain imbedding dishes--several sizes--for sorting and examining insects preserved in alcohol.
- Turntable for ringing slides.

Mounting Media

Thaxter used glycerol plus a small amount of eosin for mounting Laboulbeniales, and many of his preparations are still in excellent condition after 60 years. Some prefer a modified glycerol solution (SR-5). Amann's solution (SR-1) is a well-known medium long used for mounting fungi, and it is very satisfactory for Laboulbeniales. The high refractive index of Hoyer's medium (SR-7) makes it unsatisfactory for mounting all except highly pigmented Laboulbeniales, but it can be used during the initial stage of the preparation of slide mounts.

Slide Ringing Compounds

Shellac ringing solution (SR-19), used by Professor Thaxter, dries within a few days, adheres firmly to glass, and will not crack or peel. Fingernail polish or other plastic materials also may be used for ringing slides. Nail polish is commonly employed for this purpose, but the quality and reliability of various brands vary greatly.

EXAMINATION OF INSECTS

Insects preserved in alcohol, however collected, must first be separated from the dirt, sand, and other debris that is inevitably present. The gross collection is placed in a flat-bottom porcelain imbedding dish (or other suitable container such as a Petri dish) and all insects carefully removed and transferred to fresh 70% alcohol. Using tweezers for easy manipulation, examine each insect carefully for Laboulbeniales. Study all parts of the body under medium or high magnification with a dissecting microscope. Some species are more or less generally distributed over the surface of the host, but many are extremely restricted.

The fungi and host are often nearly the same color. In many instances the fungus is closely appressed to the integument and, if growing among setae or hairs, may be very difficult to distinguish. The blackened foot cell so characteristic of most species may serve to mark the site of an otherwise inconspicuous parasite, especially on the body of a light colored host. Parasitized insects should be stored in 70% alcohol, along with complete collection data.

PREPARATION OF SLIDE MOUNTS

Preliminary Examination

Study the parasitized insect carefully under the dissecting microscope and bear the following points in mind.

- The insect may be infested by more than one species of fungi. Thus, if several groups of closely associated individuals are present on different parts of the integument, each group may represent a separate species. Prepare several slides, if possible, from each group and take care not to mix individuals from different groups. To insure against mixing species, rinse the insect in alcohol after preparing slides of one species before proceeding with the preparation of slides of the next species.

- In order to study the development of a given species, immature as well as mature individuals of the fungus should be included in slide preparations. When the fungi are being detached from the host, make sure to remove young as well as fully developed individuals if both are present. The immature stages will ordinarily be much less conspicuous than the mature.

Procedure

- Transfer the parasitized insect from alcohol to a large concavity slide containing sufficient glycerol-chloral hydrate solution or Amann's solution to cover the insect (use solutions lacking dyes). The relatively high viscosity of these media facilitates handling not only the insect but also the parasites, once they have been removed from the host.
- Using the nondexterous hand, grasp the insect with fine tweezers and very carefully detach the parasites with a minuten needle-match stick combination held in the dexterous hand. This manipulation requires great care in order to remove the fungi undamaged, especially immature individuals. Also, one should make every effort to keep the host intact for future reference and identification.
- The fungi may be mounted immediately or transferred temporarily to a small drop of mounting fluid in a depression microscope slide.
- Using a minuten needle, center a tiny drop (only a small fraction of 1 mm in diameter) of Hoyer's medium about one-third the distance from one end of a clean slide. Place this slide and the depression slide containing the specimens side by side on the stage of the dissecting microscope so that, using the nondexterous hand, the drop of Hoyer's medium and the specimens may

be brought into view alternately by moving the slides vertically up and down.

- By means of a minuten needle, carefully transfer the fungi to the drop of Hoyer's medium. The specimens then should be positioned upside down so that they will appear erect when viewed with the compound microscope. Also, by utilizing a bit of the Hoyer's medium already present, one or two paper fibers (from lens tissue) may be affixed to the slide a short distance above or below the specimens. This prevents undue flattening and distortion of the fungi when the cover slip is added.
- Center a small drop of mounting medium on a clean 18 mm circular cover slip. Invert the cover slip and carefully lower it into position on the specimens held in place by the Hoyer's medium.
- Within a few days the Hoyer's medium will have diffused into the mounting solution and the specimens will have regained approximately normal size. This may be hastened by placing the slides for a few hours on a warming table set at 45-50°C.
- Ring the cover slip, set the preparation aside for about four days and ring again. Well-ringed slides should last indefinitely. They should be stored flat.

15 Loculoascomycetes

The Loculoascomycetes have bitunicate asci borne in a stroma in which there is no separable wall surrounding the centrum. This situation is explained by the fact that the stroma arises from the repeated division of cells of the vegetative hyphae, forming a mass of pseudoparenchyma tissue, and that the asci form directly inside this mass.

There are certain differences between the pseudoparaphyses that characterize the loculate order Pleosporales and the apical paraphyses that characterize the Hypocreales. The former arise from stromal cells at the apex of the ascostroma and grow irregularly downward. As the ascocarp matures, they become intertwined and the tips push into the underlying stromal tissue, so that free tips are usually not very evident. Apical paraphyses, on the other hand, originate as a meristematic tissue that forms in the innermost layers of the wall of the young perithecium. Their downward rate of growth is extremely uniform, producing a distinct layer or palisade of vertical hyphae, with clearly evident free tips. In certain hypocreaceous species, the cells of fully elongated apical paraphyses will swell and push together, giving the appearance of pseudoparenchyma tissue in the centrum. Both types of paraphyses are best demonstrated in young ascocarps, before the asci have elongated. (See also Müller and von Arx, 1950; Luttrell, 1955, 1965.)

Myriangiales

Myriangium, the commonest genus in the order, is found on scale insects in most parts of the United States. It has been most frequently reported on scales infesting species of *Nyssa* and *Quercus*. Stromata containing mature asci can be collected in late fall or early spring. They are small and must be searched for diligently. Since they cannot be grown in culture, dried material and prepared slides must be used in class.

Of particular interest in the Myriangiales, many of whose genera are found in warm regions, is the scattered arrangement of the ascal locules in the stroma, presumably indicative of a primitive ascostroma. Both this character and the globose, bitunicate asci show up well in prepared slides.

Elsinoe, another genus in this order, also shows these characters but is more difficult to collect. It is more common in the conidial than in the ascus stage. Some species cause anthracnose diseases of *Pyrus*, *Malus*, *Citrus*, and other hardwoods.

For additional information, see Miller, 1938, 1940; von Arx, 1963.

Pleosporales

The distinctive characteristics of this order are the bitunicate ascus and the pseudoparaphyses that grow downward into the centrum. The most common genera are *Pleospora*, *Leptosphaeria*, *Sporormia* (Pleosporaceae), and *Venturia* (Venturiaceae).

Pleospora and *Leptosphaeria* are large genera that occur mainly on herbaceous dicots and grasses. They can be collected in the fall or often in early spring on dead stems, appearing as small black dots to the naked eye. With some experience they can be recognized, as a group, with a hand lens, but microscopic examination is necessary to separate them positively from pycnidia or perithecia of similar fungi. *Pleospora herbarum* is one of the most common species, occurring on herbaceous stalks of many plants from alpine and arctic to warm temperate regions. Many species require overwintering before mature ascospores are formed. Material collected in autumn will usually best demonstrate the pseudoparaphyses, which sometimes disintegrate as the asci mature. Some species produce conidia in culture but rarely form ascostromata. Thus dried material is more likely to be suitable for class use.

Pleospora includes easily collected examples of the order. The genus also is represented in major culture collections. *Stemphylium* conidial states of several species of *Pleospora* can be found at any time on current season growth of hosts, immature ascostromata by autumn and mature ascospores by spring. Overwintered *Allium*, *Gladiolus*, and *Lycopersicon* leaves and stalks are likely sources of species that will produce both conidial and ascocarpic states in culture. Lacking a natural source of easily identified species, it is best to use pure strains of *Pleospora herbarum* (conidial state = *Stemphylium botryosum*) or of the more common and often more quickly maturing *Pleospora* state of *S. vesicarium*. Inoculate hay decoction agar (M-12) and potato-carrot agar (M-17) in slanted tubes and in Petri dishes poured at least half full. Incubate at about 25°C for two weeks, by which time conidia will be abundant and small stromata visible. Relocate tubes and plates in a relatively humid incubator at 15-20°C; alternatively, place cultures in 5-7°C refrigerator. After two weeks at either temperature the largest of the ascostromata will contain well-defined pseudoparaphyses. Within six weeks there will be a few asci; those formed at the higher temperature will contain very young to fully developed ascospores. Beginning at about eight weeks, mature asci can be found in refrigerated cultures. By this time the bitunicate nature of the wall will be strikingly apparent in both intact and ruptured asci. Depending on the isolate, maturation of significant numbers of ascospores may not occur before three months or more of growth, particularly at refrigerator temperatures. For this reason, incubation of cultures at 15-20°C is to be preferred if sufficient humidity to prevent drying of cultures can be maintained (See also Simmons, 1969).

Sporormia, which is sometimes placed in a family of its own, is common on various kinds of dung. Several species are readily obtained from dung kept a week or more in moist chamber, produce ascocarps readily in culture, and make excellent material for demonstrating pseudoparaphyses and the extension of the inner wall of the bitunicate ascus when mounted in water. The large brown ascospores aid in observing the bitunicate wall, as ascospores are discharged under the microscope. Mature ascocarps are formed in 14 days.

The family Venturiaceae includes a number of pathogens and many saprophytes, the majority of species having green, one-septate ascospores. *Venturia* spp. are common on overwintered leaves of many deciduous trees and shrubs, e.g., *V. inaequalis* on apple, *V. pirina* on pear, *V. ditricha* on birch, *V. alnea* on alder, *V. macularis* on poplar, and many more. The conidial stages of these are pathogenic on living leaves, often forming spots. Species of *Gibbera* and *Antennularia* appear to occur most commonly on members of the Ericaceae and Rosaceae. Ordinarily these fungi mature after overwintering. Species of *Coleroa* (*Stigmatea*) mature on living leaves during summer and fall. *C. robertiana* on *Geranium* species in the east, *C. circinans* in the west, and *C. rubicola* on *Rubus* are common and widespread in North America. *Lasiobotrys* is conspicuous on living *Lonicera* leaves and *Rhizogone* on *Symphoricarpos* in western North America. *Phaeocryptopus* species occur on *Abies*, *Pinus*, and *Pseudotsuga* needles, the small, glabrous ascostromata forming superficially in rows on lower needle surface, seated on a foot-like structure that penetrates the host through stomata. *Apiosporina* is now included in this family; the two species *A. morbosa* on *Prunus* and *A. collinsii* on *Amelanchier* are common throughout temperate North America.

For additional information, see Arnold, 1928; Keitt *et al.*, 1943; Wehmeyer, 1955, 1961; Holm, 1957; and Kerr, 1961.

Dothideales

Members of the Dothideales have a pseudoparenchymatous centrum and bitunicate asci. They are distributed widely, many causing diseases of crop plants. Two very common genera are *Mycosphaerella* and *Guignardia*. *Mycosphaerella* occurs on a wide variety of herbaceous and woody plants, may be found in spring on overwintered leaves in north temperate regions, and is frequently the cause of serious leafspot diseases. *Guignardia* causes leaf diseases on members of the Vitaceae. Both genera can be collected in late summer or early autumn on fallen leaves; leaves with leafspots are likely to contain ascostromata. On stems small black dots may indicate ascostromata, but microscopic examination is necessary to determine this for certain. Ascostromata at this stage will contain young asci and show the pseudoparenchymatous centrum.

As leafspot fungi, the conidial stage will be frequently encountered. Isolations may be made from conidia. Another technique is to isolate the fungus directly from the host tissue. This may be done by cutting out small squares of leaf tissue containing the spot. These squares are then sterilized in a 10% aqueous (or alcoholic) solution of sodium hypochlorite (commercial chlorox) for about two minutes. The squares are laid on a nutrient agar such as V-8 (M-29), after which the fungus will grow out onto the agar.

The imperfect stages of many species of *Mycosphaerella* can be grown in culture but seldom produce ascocarps. One species, however, *M. citrullina*, produces ascostromata readily in culture. In nature it is usually the imperfect stage, *Ascochyta cucumis*, that is found. Isolations can be made from conidia; a number of isolates should be made, as they vary in their tendency to form ascostromata. Pycnidia will form in about five days in culture, and form long cirrhi when mounted in a drop of water under a loose cover slip. Cultures grown on cellulose squares will show various stages in the development of stromata from vegetative mycelium at this time. There does not appear to be any sure way of distinguishing between pycnidial and ascostromal initials, since both form in the same manner. After six days, cultures will have small ascostromata containing young asci. This stage is particularly good for showing young asci pushing up through the pseudoparenchymatous centrum. At eight days mature ascospores will have formed, and the extension of the inner wall of the ascus can be demonstrated by mounting them in a drop of water. The cover slip should be pressed just enough to squeeze out the asci. Ascus expansion frequently occurs after a few minutes.

The conidial stages of the different species are diverse, some being hyphomycetous and others forming pycnidia or acervuli. If several species of the genus are available, this diversity can be demonstrated to the student.

Guignardia does not produce ascostromata in culture, so dried or fresh collections must be used.

Leptosphaerulina is another fungus that shows the characters of this order well. It produces leafspots on several legumes, such as clover and peanuts, as well as on a number of other crop plants. Species of *Leptosphaerulina* form ascostromata readily in culture. In *L. trifolii* young ascostromata with ascogenous cells are formed in seven days. The various stages of stroma formation can also be clearly seen at this time simply by scraping mycelium off of the surface of the agar and mounting in water. Cellulose squares work well too. Young stromata are composed of hyaline cells, the hyphae becoming dark brown in older mycelium. Mature asci are produced in 14-17 days.

Species of *Dothidea* produce a pulvinate stroma that contains many locules, e.g., *D. ribesia* on *Ribes* branches, *D. sambuci* on many woody hosts. Ascospores of both genera will germinate readily, but production of ascostromata is more difficult to achieve.

For additional information, see Higgins, 1936; von Arx, 1949; Luttrell, 1951b; Loeffler, 1957, and Barr, 1958.

Hysteriales

These fungi, which traditionally have been placed among the Discomycetes, are interesting for their elongated, boat-shaped ascocarps. Because those that have been studied possess bitunicate asci and a pseudoparaphysate centrum, they are now considered to be related to the loculate Ascomycetes. They occur widely on decorticated dead wood and are often abundant on discarded posts or lumber that is well weathered, often occurring in large groups. *Hysterographium*, *Hysterium*, and *Glonium* are commonly encountered in the field. Mature specimens can be collected in the autumn on bark of living trees and rotting logs of both conifers and hardwoods. The ascocarps, which are sometimes branched, have a slit along the top.

The conidial stages of several species have been obtained in culture, but no ascocarps form. Thus dried or freshly collected material must be used.

For additional information, see Lohman, 1933; Bisby and Hughes, 1952; Luttrell, 1953.

Microthyriales

The majority of the Microthyriales seem to be subtropical to tropical. The flattened, inconspicuous ascostromata of several species can be found in north temperate retions on such plants as *Gaultheria*, *Linnaea*, several conifers. (See also Stevens and Ryan, 1939; Luttrell, 1946.)

16 Fungi Imperfecti

Because it is unlikely that examples of all genera considered in this chapter will be found in any one geographical area, it is wise to maintain a representative set of cultures that have been identified by specialists and that are known to act in predictable ways under laboratory conditions. Field collections and laboratory isolations and identifications can be made of genera easily found in a given region, and these substituted, as available, for the pure cultures.

SOURCES OF MATERIAL IN THE FIELD

Representatives of the Fungi Imperfecti can be obtained at any season, in practically any habitat, and on all substrates of organic origin that have not been naturally or artificially protected. This is not to say that a given species can be located at will. But the number of genera that can be obtained within a week is enormous, given only a couple of grams of garden soil, a few ml of sewage, sewage-polluted water, or organically enriched stream-side soil, a few overwintered or weathered leaves, stems, and fruits, two or three dead insects, and a couple of pieces of paper or cloth decaying on the ground. An overwintered leaf, pressed gently to the surface of hay agar in a Petri dish, will yield a score of species in a dozen different genera.

The microscopic size of most of the imperfects makes field determination, even to genus, virtually impossible. When spores are present on a collection, it is best to make slide mounts of only a small portion of the material for initial study, proceeding at once to isolation of spores on agar plates.

A primary technique in obtaining sporulating specimens is the use of a moist chamber. Sterile Petri dishes containing a small wet wad of absorbent cotton at one side are excellent for most small specimens; larger covered containers are suitable, provided a moderately high internal humidity can be maintained. But in "collecting" imperfects in a moist chamber, caution must be exerted to examine specimens frequently (at least daily) so that the rare, unusual, or slow-growing species can be discovered and isolated before they are overgrown by the common weed species. "Collecting" from soil, sewage, dung, and similar material that carry high populations of bacteria usually requires techniques that involve dilution plates and antimicrobial agents.

Collecting a given pathogen at will (if an appropriate host is available) is somewhat easier than is collecting a particular saprophytic species. Two good guides to fungus species known to occur on plants in North America are the Seymour *Host Index* (1929) and the excellent, annotated U.S.D.A. *Index of Plant Diseases* (1960).

The following list of habitats and physiological niches is augmented by references to helpful literature.

Aquatic Forms

The hyphomycete flora to be found in fresh-water habitats can be examined on collections of plant and insect debris. Many of the known species do not sporulate freely, but in any event watch for the rather bizarre branching of spores in many species of this group as an aid in locating them on substrates and in slide preparations. Phloxine is suggested as a suitable mounting medium. (See Ingold, 1942, 1943, 1944, 1949, 1952, 1958a,b; Ranzoni, 1953; Petersen, 1962, 1963.)

Cellulolytic Forms

Large numbers of the imperfects are active in the degradation of cellulose in nature. A good place to look for these is on decaying cotton fabrics and paper in contact with the soil or in moist places. The fact that a given fungus is found on such a substrate is not, however, proof that it is cellulolytic, since contaminating soil, dung, oils, and fillers used in manufacturing processes may be the true food source. (See Reese *et al.*, 1950.)

Coprophilous Forms

Any fecal pellet that has been in contact with the soil will yield imperfects in a moist chamber. Often it is more informative to break open fresh pellets aseptically and to incubate them or to work the interior material that has not been contaminated with soil through dilution series. (See Tubaki, 1954.)

Entomogenous Forms

While the body of any dead insect kept in a moist chamber will give rise to colonies of whatever spores contaminate the surface, examples of truly entomogenous species should be looked for on debilitated or dead insects and larvae that have fungi already sporulating on them at the time of collection. A useful technique is to surface-sterilize the insect body and then hold it in a moist chamber or plate it on a suitable agar medium, watching for fungi that grow out through the body walls (Petch, 1948; Mains, 1948, 1955; MacLeod, 1954a, b).

Fungicolous Forms

The surfaces of aging or overwintered agarics and polypores often have abundant colonies of hyphomycetes sporulating on them; most of these are readily observed and easily isolated. The study of imperfects that are truly parasitic on other fungi has scarcely been started; they are probably far more widespread than the literature indicates. In routine isolations, watch for sporulating structures of one species on hyphae of a second species that has been reisolated several times by means of single-conidia or of hyphal tips. Mixed cultures of two or more species that simply grow together do not meet these criteria. (Gordon, 1944-59; Tubaki, 1955; Nicot, 1962; Barnett and Lilly, 1962; Barnett, 1964b)

Graminicolous Forms

The economic value of the cereals and other grasses has led to abundant literature on both their parasitic and their saprophytic fungal floras. Collections of sporulating imperfects in heads, leaves, and stalks can be made easily or can be obtained from such materials held in moist chambers. A great many of the graminicolous species grow readily in culture. (Sprague, 1950)

Marine Forms

Submerged wood and other plant debris are good sources. Many "terrestrial" species that can tolerate the salt concentration of sea water are to be expected. But a few species are known that are physiologically restricted to marine or at least brackish water habitats. Isolates of these latter usually require media prepared with natural or artificial sea water. (Barghoorn and Linder, 1944; Moore and Meyers, 1959; Meyers and Moore, 1960; Siepmann and Johnson, 1960; Johnson and Sparrow, 1961)

Plant Pathogens

Thousands of names have been erected for imperfect species solely on the basis of their association with certain hosts, thus often giving rise to a plurality of names for a relatively few common species. But the fact remains that the easiest way to collect representatives of this group is through recognition of specific diseases. Leaf spots, inflorescence blights, root rots, wilts, discolorations, and the like are guides to the possible presence of pathogenic imperfects, either sporulating in place or capable of being developed in a moist chamber. Parasitism and pathogenicity cannot be simply assumed, of course. (Seymour, 1929; U.S.D.A., 1960; Walker, 1969)

Species from Sewage and Polluted Water

Collection of imperfects from these habitats is essentially a matter of isolation from dilution plates. The presence of enormous numbers of bacteria in samples makes it essential to use isolation media containing antibacterial agents, e.g., rose bengal-aureomycin agar (M-55). A yeast nitrogen-base, glucose broth, in a shake culture technique, will yield a variety of yeasts previously unsuspected of occurring in organically enriched waters, soils, and other habitats. (Cooke, 1954, 1957, 1965)

Species from Soil

As a reservoir of organic debris in all states of deterioration, soil samples can be expected to yield considerable numbers of fungi. Soil streaked on the surfaces of agar plates will give plenty of material for study, but mixed colonies and bacterial contamination will make isolation work and critical study difficult. Dilution plates and media containing antibacterial agents (e.g., rose bengal-streptomycin agar) will facilitate the development of mold colonies without undue crowding and massive bacterial contamination. (Gilman, 1957; Burges, 1958; Barron, 1968; Domsch and Gams, 1970)

Species from Self-heating Organic Matter

Imperfects that develop readily at temperatures near 50°C may be found in such self-heating accumulations of plant material as compost heaps and wet hay. Representatives of the group may occasionally be found sporulating in nature; usually, however, it is necessary to develop colonies under controlled conditions. High temperatures must be maintained in order to discourage mesophilic weed molds; high humidity must be maintained in order to prevent drying of agar media. (Cooney and Emerson, 1964; Crisan, 1964; Fergus, 1964)

CULTURING

Methods and media suitable for initial isolation work or for production of typical sporulating structures are not necessarily suitable for comparison and identification work or for conservation of stock cultures.

Production of Typical Sporulating Structures

As a general rule, low-nutrient media are recommended for sporulation. There are numerous exceptions, particularly among the plant pathogens, for which media containing sugars (sometimes in considerable concentration), starch, or proteinaceous material may be necessary. A reasonable range would cover potato glucose (M-18), malt extract (M-48), cornmeal (M-5), Czapek's (M-89), V-8 juice (M-29), and peptone-yeast extract (M-51) agars. Should none of these prove adequate, the best advice is to search the literature on the genus being studied for more explicit recommendations.

Most of the commonly isolated Imperfecti (particularly the saprophytes) will sporulate at moderate temperatures in the dark or at least in the diffuse light of the laboratory. But many sporulate better, or only, when grown at least part of the time in fairly strong light. It is a good practice to expose nonsporulating colonies to full sunlight for 15-20 minutes, to ultraviolet light for 2-3 minutes, or to a few hours of near UV before deciding to move on to the more esoteric media; each period of light exposure should alternate with a period of darkness or subdued light. The effect on sporulation within the next 1-2 days after exposure often is dramatic.

The "near-UV" technique for inducing sporulation is highly recommended because of its effectiveness, simplicity, and low cost. A suggested system includes use of "Black Light" fluorescent lamps ("cool white daylight" fluorescent lamps emit sufficient near-UV to induce sporulation in many species); an alternating cycle of 12 hr near-UV and 12 hr dark starting 3-4 days after inoculation of plates (plastic or Pyrex); and temperatures controlled near 21°C or, if possible, fluctuating from 18°C in the dark period to not higher than 24°C in irradiation period. See the Plant Pathologist's Pocketbook (Commonwealth Mycological Institute, 1968) for construction details of the system used effectively by C.M.I. for a wide range of organisms. A comprehensive review of the subject has been written by C. M. Leach (1971).

Some habitat groups of imperfects have culture requirements that should be recognized whenever they are handled for sporulation. The aquatic forms, for example, have relatively low optimum temperatures for growth; a range of 15-20°C is recommended as a departure point. In addition, these aquatics often do not sporulate in pure culture until they are submerged in water that is strongly aerated. Grow strains on oatmeal agar in Petri dishes until colonies reach 1.0-2.0 cm in diameter. Cut the colonies into several strips (sterile knife) and submerge them in sterile distilled water in large test tubes; maintain at 15°C with strong aeration (sterile glass tubing attached to a source of filtered air). Examine for sporulation over a period of 3-14 days (Petersen, 1960).

In the primary isolation of yeasts using liquid culture techniques, purification of isolates is critical. After 64-80 hours incubation in shake culture, the growth is allowed to settle and loopfuls are then streaked on yeast extract-malt extract-glucose agar (M-67), or Diamalt agar. After two or three days, colonies are picked or areas of streaks are picked and restreaked. Pure cultures are transferred to neopeptone-dextrose agar slants, or malt agar slants, for storage until a convenient time to effect identification.

Thermophilic imperfects must be cultured at elevated temperatures (40-50°C); many of them will not grow at all at moderate temperatures. Extra care must be taken to insure high humidity around these developing cultures in the incubator; otherwise the agar substrates and colonies will dry rapidly and become useless for comparison and conservation purposes.

Comparison and Identification of Isolates

There is no such thing as a standard medium or set of media suitable for comparison and identification work. The best advice at the outset is to use for isolates of a given genus those media that have been reported in monographic studies or major literature on the genus. Lacking such guidelines, low-nutrient media or media containing sterile plant parts and extracts similar to those present in the original substrate are recommended. There is little point in attempting to identify one or two isolates of a genus on high-nutrient media that enhance characters that may be quite unobservable in nature and completely unreported in the taxonomic literature.

Culturing for Conservation

A set of pure, stable, identified stock cultures is a tremendous aid to identification and experimental work. The general rule in choosing conservation media is to use the medium that previous work in Petri dishes has shown will support moderate growth with abundant sporulation; always choose a medium that enhances sporulation rather than production of vegetative mycelium.

SYSTEMS OF CLASSIFICATION

The Saccardo system has long been in use for the classification of the imperfects. The primary basis of the system is the morphology of the mature sporulating structures as they are known in nature. Emphasis is also placed on spore morphology and color. See Alexopoulos (1962) or Bessey (1950) for an outline of this system, and Saccardo (1882-1931) for greater detail. The Saccardo system is at present the only comprehensive scheme in which a niche has been found for each of the thousands of described genera. However, in recent years a more nearly natural scheme of classification that shows great promise has been in the process of development.

The "Hughes system" had its evolutionary roots in the systematized opinions of earlier mycologists, but it has been developed only recently by Hughes (1953), Tubaki (1963), and Barron (1968). This scheme is based on basic types of conidiophore-conidium development and has to date been applied to the Hyphomycetes (Moniliales). Conidium and conidiophore morphology and color are used as secondary characters in classification.

CULTURES FOR CLASS USE

For various reasons, it would seem best to present the imperfect fungi to the class in general mycology as a series of representative genera in each of the groups.

Fresh or dried material may be used for many of the genera, especially those on leaves or wood, but rarely are all stages of development present. It is best to use cultures of selected known genera to supplement or replace those found in nature. It would seem desirable to have available several more genera than are expected to be used, for contamination and failure to sporulate are frequent.

The instructor is encouraged to maintain a set of teaching cultures from year to year. Most species can be maintained for several months on agar slants held at 5°C. Many imperfects sporulate more quickly and sometimes more abundantly on a weak medium, such as glucose 1-2 g and yeast extract 1 g per liter. Some people prefer an infusion of hay, cornmeal or other natural product. The amount of mycelium can be controlled by the amount of available carbon source in the medium. A richer medium, such as glucose 5-10 g and yeast extract 2 g per liter, is often more satisfactory for production of pycnidia, acervuli, or sclerotia.

It is suggested that both a weak and a rich medium be used for most genera unless they have been tested before use in class. Most genera should be incubated near 25°C for rapid production of conidia. Some light should be provided, such as alternate light and darkness in the laboratory or culture room. Inoculation may be at one or several points on the agar plate, but cultures will remain usable longer if inoculation is at one point near the edge of the dish. Most genera produce spores in 3-10 days and all should be checked before use in class because of the variation in species or isolates.

GROUPS AND REPRESENTATIVE GENERA

Generic examples are listed for the four ordinal groups: Moniliales, Mycelia Sterilia, Melanconiales, and Sphaeropsidales. Since the "Hughes system" has been tested for increasingly large numbers of genera among the Moniliales, a series classification based on this system (Barron, 1968) is used in listing representatives of this order. The types of conidial ontogeny in the other orders are noted for those genera in which they are known. Conidium form and color are given for each genus.

Additional notes on sources, handling, and class presentation of each genus marked with an asterisk are consolidated in a subsequent section entitled "Genera Recommended for Study," p. 219.

The well-known form genera *Aspergillus* and *Penicillium* are discussed at considerable length (p. 231) in order to present recommendations on isolation, cultivation, examination, etc., that may be used or modified for use with great numbers of other genera of Imperfecti.

The following general reference works will be helpful: Saccardo, 1882-1931; Hughes, 1953; Alexopoulos, 1962; Tubaki, 1963; Barron, 1968; von Arx, 1970; Ellis, 1971; Barnett and Hunter, 1972.

Moniliales

Conidial fungi without pycnidia or acervuli. Conidiophores absent or well developed, separate, clustered, or compacted into sporodochia or synnemata.

Series Arthrosporae Conidia formed by septation and breaking up of segments of simple or branched hyphae, which may be vegetative in origin or formed on distinct conidiophores. (Figure 4a-b)

**Geotrichum* (hyaline, 1 cell)
**Oidiodendron* (hyaline, 1 cell)
Amblyosporium (hyaline, 1 cell)

Series Meristem Arthrosporae Conidia develop basipetally, originating from the meristematic apical region of the conidiophore; the youngest conidium of a chain scarcely differing from the conidiophore, and successively formed conidia incorporating the tip of the gradually shortening conidiophore. (Figure 4c)

**Acrosporium* (hyaline, 1 cell)
**Trichothecium* (hyaline, 2 cells)

Series Aleuriosporae Conidia produced singly, rarely in chains, developing terminally as swollen ends of conidiophores or sporogenous cells, commonly thick walled and pigmented but some thin walled and hyaline. (Figure 4d-f)

**Humicola* (dark, 1 cell)
Sepedonium (hyaline, 1 cell) Large yellow aleuriospores usually accompanied by a phialospore state.
**Chrysosporium* (hyaline or brightly colored, 1 cell)
Pithomyces (dark, several cells)
**Epicoccum* (dark, dictyospores)
Microsporum (hyaline, phragmospores) Several species, but not all are pathogenic to man and animals. *M. gypseum* (dermatophytic) cultures easily and sporulates freely.

Series Annellosporae The first conidium forms as an apical aleuriospore; successive conidia are formed as swollen ends of conidiophores or sporogenous cells through the previous conidial scars, resulting in a close series of indistinct annellations in the apical region of the sporogenous cell. (Figure 4g-h)

**Scopulariopsis* (hyaline to dark, 1 cell)
**Trichurus* (dark, 1 cell; synnematous)
Spilocea (dark, 1-2 cells) Conidial state of *Venturia inaequalis*, shows series characteristics well.

Series Porosporae Conidia develop through pores at the apex or side of the conidiophore, often thick walled; conidiophores may be determinate in length or may continue growth either through the apical pore or by sympodial extension from a lateral point near the apex. (Figure 4i-j)

*_Alternaria_ (dark, dictyospores; solitary or acropetally catenulate)
*_Bipolaris_ and _Drechslera_ (dark, 3-several cells)
Curvularia
Torula (dark, 1-several cells; phragmospores)

Series Sympodulosporae Conidia develop as swollen ends of conidiophore or of sporogenous cell (not from pores) and form on successive new growing points arising below the previous conidia; conidiophore or sporogenous cell increasing in length or becoming swollen. (Figure 4k-m)

*_Beauveria_ and _Tritirachium_ (hyaline, 1 cell)
*_Arthrobotrys_ (hyaline, 2 cells)
Calcarisporium
Piricularia
*_Cercospora_

Series Blastosporae Conidia develop as swollen ends of conidiophores or sporogenous cells or as buds from existing conidia; solitary or frequently in simple or branched acropetal chains; conidiophores simple, branched, or lacking as a distinct structure. (Figure 4n-o)

Cryptococcus, _Rhodotorula_, or other asporogenous yeast
*_Aureobasidium_ (hyaline to slightly pigmented, 1 cell)
*_Monilia_ (hyaline, 1 cell) Conidial state of _Monilinia_ spp.
*_Cladosporium_ (hyaline to dark, 1-2 cells)
Bispora (dark, mostly 2 cells)
Periconia (dark, 1 cell)

Series Botryoblastosporae Blastospores develop on swollen sporogenous cells (ampullae) that produce several conidia more or less simultaneously; conidia may be solitary on conspicuous denticles or may give rise to simple or branched acropetalous chains; series of ampullae or sporogenous nodes may form through proliferation of the conidiophore. (Figure 4p-q)

Oedocephalum (hyaline, 1 cell)
*_Botrytis_ (hyaline or gray, 1 cell)
*_Botryosporium_ (hyaline, 1 cell)

Gonatobotrys (hyaline, 1 cell) *G. simplex*, parasitic on *Alternaria* or *Cladosporium*, is a good example of ampullar nodes.

Gonatobotryum (dark, 1 cell) *G. apiculatum* recommended as example; *G. fuscum* is parasitic on *Ceratocystis* spp.

Series Meristem Blastosporae Blastospores borne singly at apex and side of conidiophores that elongate from a meristematic base. (Figure 4r)

**Arthrinium* (dark, 1 cell)

Series Phialosporae Conidia are cut off successively from the open growing point at or within the apical portion of the sporogenous cell (phialide), sometimes remaining in basipetal dry chains or often being held in droplets of slime; apex of phialide may flare as a collarette or may be tubular or relatively undifferentiated; usually the phialide does not increase in length; phialides may be subcylindrical, flask-shaped, or with a distinctly swollen base; distinct conidiophores may be present or absent. (Figure 4s-u)

Thielaviopsis, *Chalaropsis*, and *Chalara* (hyaline, 1 cell) Dark aleuriospores also present in first two genera.

**Phialophora* (dark, 1 cell)

Verticillium (hyaline, 1 cell)

**Gliocladium* (hyaline, 1 cell)

Penicillium (hyaline or bright color, 1 cell) See extended treatment following.

Aspergillus (variously colored, 1 cell) See extended treatment following.

**Fusarium* (hyaline, 1-several cells)

Stilbum (hyaline, 1 cell; synnematous)

Cylindrocladium (hyaline, 2-6 cells)

**Trichoderma* (hyaline, 1 cell)

Mycelia Sterilia

Conidia not produced; chlamydospores, irregular clusters of cells (bulbils), and distinct sclerotia may be produced.

**Sclerotium*

Rhizoctonia

Melanconiales

Acervuli usually well formed on host in nature; in culture the conidiophores may be separate, clustered into flat or cushion-shaped masses resembling sporodochia, or enclosed in so much sterile tissue as to be pseudopycnidial.

- **Colletotrichum* (hyaline, 1 cell, phialospores)
- *Melanconium* (dark, 1 cell, annellospores)
- **Pestalotia* (dark, phragmospores, annellospores)
- *Cylindrosporium* (hyaline, scolecospores)
- *Coryneum* (dark, phragmospores)
- *Steganosporium* (dark, dictyospores)

Sphaeropsidales

Conidia produced in pycnidia that may develop singly or in groups, sometimes on or in a stroma; pycnidia usually well formed in culture, but stromatic tissue may be poorly formed or lacking.

- **Phoma* (hyaline, 1 cell, interpreted by different authors as blastic, phialidic, or annellidic in origin)
- *Sphaeropsis* or *Coniothyrium* (dark, 1 cell)
- *Cytospora* (hyaline, 1 cell) Pycnidia in stroma.
- *Darluca* (hyaline, 2 cells) Parasitic in rust pustules.
- **Diplodia* (dark, 2 cells)
- *Septoria* (hyaline, scolecospores)

GENERA RECOMMENDED FOR STUDY

Acrosporium

Worldwide; parasitic on wide range of wild and cultivated plants as the "powdery mildew" oidial state of Erysiphaceae; common on leaves of grape, rose, lilac, clover, cucurbits, cereals, and wild grasses. Obligate parasites; fresh material on living leaves left overnight in moist chamber usually is excellent for observation. The distal (older) conidia detach very readily. Strip epidermis from infected host, mount in drop of wetting agent, and stain in phloxine. Extensive search

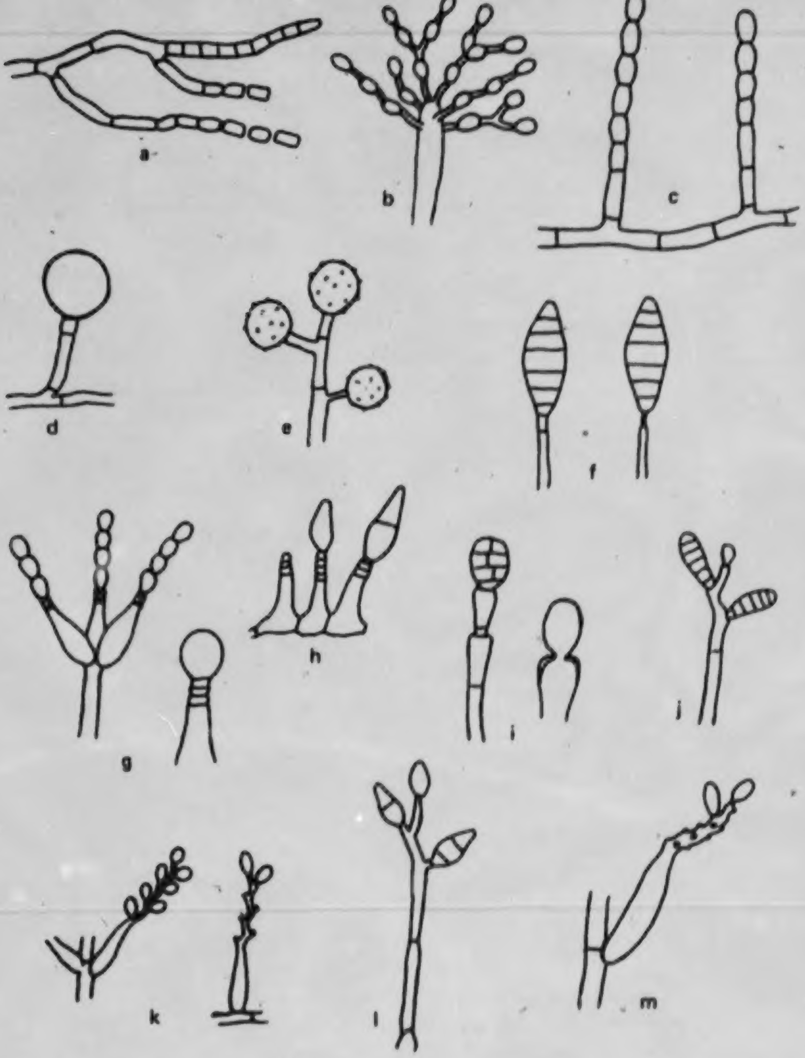
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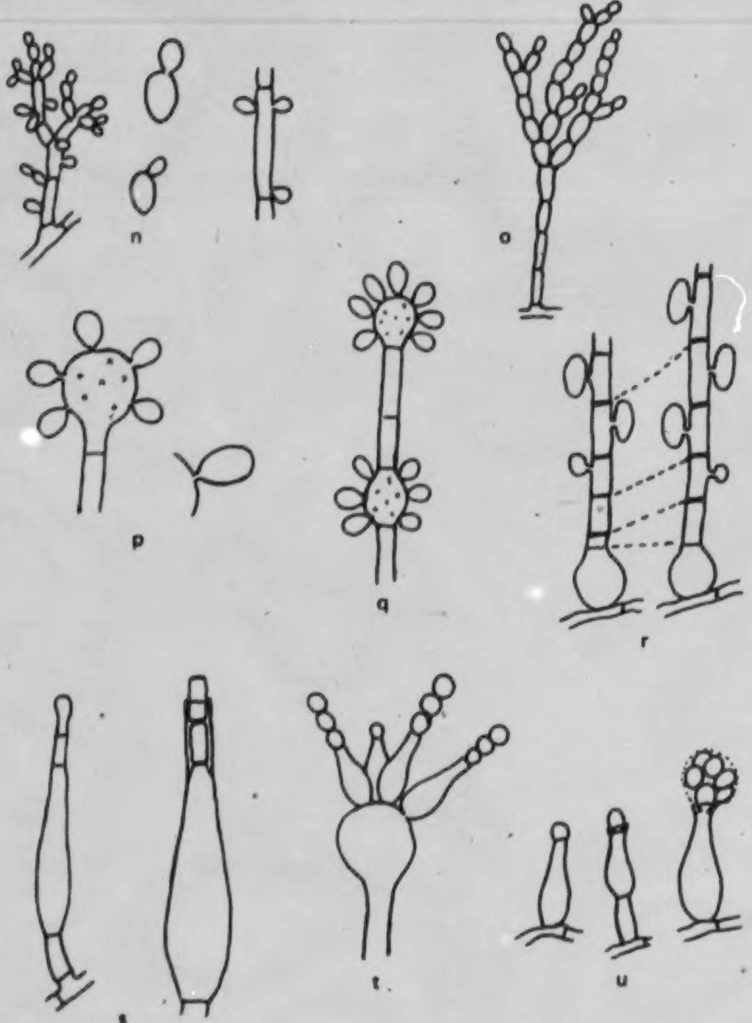
FIGURE 4 *Sporulation in Fungi Imperfecti.*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is often necessary to locate intact chains of conidia. (Sprague, 1950; Walker, 1969)

Alternaria

Cosmopolitan; ubiquitous saprophytes on debris of herbaceous plants; weak parasites. Several are common pathogens on herbaceous crops and related weeds (e.g., leaf spots of cucurbits, potato, tomato; leaf spot and flower blight of zinnia). Overwintered or weathered leaves and stems commonly yield saprophytic species in moist chambers. Easily cultured, but good sporulation of pathogens requires light exposure (place 3-4 day-old cultures in sunlight for 30 min; or alternate 12 hr near-UV with 12 hr dark). Examples: *A. tenuissima*, hay agar (M-13), excellent; *A. cucumerina*, V-8 (M-30) or potato carrot (M-17), fine after light or near-UV on 3-4th day. (Groves and Skolko, 1944; Neergaard, 1945; Joly, 1964)

Arthrinium

Widespread; saprophytic, commonly in patches on dead leaves and culms of grasses and sedges, often in moist or marshy habitats late summer and fall. Example: *Arthrinium* state (syn. *Papularia arundinis*) of *Apiospora montagnei*, 1-2 weeks on potato glucose agar (M-18). (Ellis, 1965)

Arthrobotrys

Probably widespread, though described mostly from North America and Europe; saprophytic in decaying plant debris or parasitic on nematodes. Often develops late (4-6 wks.) in culture plates seeded with well-decayed plant debris. Isolate from aerial spores to avoid bacterial and fungal contamination; cornmeal agar recommended. Tends to be difficult to maintain in pure culture; nematode-trapping structures (hyphal rings and pegs) are absent in pure culture on common media, but should be sought in nematode-infested plates. Example:

Arthrobotrys spp. (or *Dactylaria* spp., *Dactyella* spp.). (Drechsler, 1937, 1950; Muller, 1958)

Aureobasidium

Cosmopolitan; most common mildew of painted surfaces; frequent component of the dark mold complex growing on the honeydew of scale insects on leaf surfaces; often in brown slime flux of elm and other trees; often associated with storage and shipping decays of fruit. Seldom specifically identifiable on field collections. Isolate from diluted or streaked scrapings and washings; watch for moist, hyaline, slowly spreading rhizoidal colonies that are highly variable in production of dark pigment. Commonly found in the literature under generic name *Pullularia*. Example: *A. pullulans*; grow 4-5 days on potato glucose agar or potato carrot agar. (Cooke, 1959)

Beauveria

Widespread but not commonly encountered; primarily parasitic on insects (e.g., potato beetles, cockchafers, silkworms, spiders, locusts, bees, wasps, various larvae and cocoons). Isolate from dead insects and mummified larvae; surface sterilize in 2-5% aqueous Na- or Ca-hypochlorite. Inoculate Petri dishes of Sabouraud maltose agar (M-46); isolate from white mycelium that develops on surface of specimen or on the agar. Culture on hay infusion agar for good conidiophores with reduced vegetative mycelium, on Sabouraud maltose agar for abundant mycelium, which sometimes becomes coremial after a few weeks. Example: *B. bassiana*. (MacLeod, 1954a, b; Kendrick and Cole, 1968)

Bipolaris

Cosmopolitan; chiefly grass parasites segregated from *Helminthosporium*; occurs on leaf spots, at culm nodes, and in inflorescences. *Drechslera* is a similar genus of grass parasites also segregated from *Helminthosporium*. Sporulation representing either genus may be present on field collections or it can be induced in a moist chamber. *Bipolaris* conidia are fusoid, germinating from two end cells; ascosporic states in *Cochliobolus*. *Drechslera* conidia are cylindric, germinating from any cell; ascosporic states in *Pyrenophora*. Most pre-1959 literature and much of later work uses the name *Helminthosporium* for this entire group. However, *Helminthosporium* correctly refers to lignicolous species related to the original type of the genus. Examples: *Bipolaris* state (syn. *Helminthosporium maydis*) of *Cochliobolus heterostrophus*; *B. sorokiniana*, culture 4-5 days on potato carrot agar. (Drechsler, 1923; Hughes, 1953, 1958; Shoemaker, 1957, 1959, 1962)

Botryosporium

Probably widespread; saprophytic on decaying plant parts, paper, and other organic debris; reported to be frequent in greenhouses. Grows moderately rapidly in culture. Example: *B. pulchrum*; culture 4-5 days on hay infusion agar. (Hughes, 1953; Barron, 1968)

Botrytis

Cosmopolitan and common; saprophytic or frequently plant parasitic; on herbaceous debris in moist habitats and on such materials held in moist chamber; common on decaying leaves, rhizomes, buds, and bulbs of iris, narcissus, onion, gladiolus, lily, tulip, peony. Conidial states of *Botryotinia* (= *Sclerotinia* subgenus *Botryotinia*). Example: *B. cinerea*; culture 5-7 days on hay infusion agar. (Hennebert and Groves, 1963; Barron, 1968)

Cercospora

Cosmopolitan parasites, commonly causing leaf spots on thousands of different host species, but also growing effusely on some hosts or on spots of other plant parts.

Sporulates on spots in nature, or spores may develop from dry spots in moist chamber. Growth and sporulation in culture usually poor. Several species are conidial states of *Mycosphaerella*. Example: *C. musae*; very poor growth on hay infusion agar, but conidia are typical. Use as much inoculum as is available when making transfers; macerate inoculum with inoculating needle and distribute widely over agar surface; expect only tiny colonies. (Davis, 1938; Chupp, 1953; Deighton, 1959)

Chrysosporium

Temperate and tropical; most species are likely to be associated with soil (especially from animal pastures and chicken yards), commercial mushrooms ("mat" disease, *C. luteum*), dung, skin and hair, decaying cotton and woolen fabrics, and feathers. One species (*C. dermatitidis = Zymonema dermatitidis*) is the causal agent of North American blastomycosis. Soil dilution plates and specimens held in damp chamber should be checked for a period of up to a month because growth rate is slow on low-nutrient media and only moderate on media containing peptones and dextrose, e.g., Phytone-yeast extract agar (M-54). Example: *C. luteum* (commonly in literature as *Myceliophthora lutea*); culture 10-12 days on hay infusion agar; growth is typical. (Carmichael, 1962)

Cladosporium

Cosmopolitan; saprophytic and parasitic; the two commonest species (*C. herbarum* and *C. cladosporioides*) are present practically everywhere, and it is almost impossible to hold pieces of plant debris in a moist chamber without this genus appearing. Parasitic species include *C. fulvum* (on leaves and fruit of tomato) and *C. cucumerinum* (on leaves and scabbed fruit of cucumber). *C. resinae* (often in literature as *Hormodendrum*) has an unusual habitat on (among other things) creosote-treated wood. A known perfect state is in *Mycosphaerella*, another in *Amorphotheca*. Example: *C. herbarum*; sporulation excellent on hay infusion or potato carrot agar in 4 days. (deVries, 1952; Barr and Tomes, 1961; Parbery, 1969)

Colletotrichum

Cosmopolitan; saprophytic or commonly related to leaf and fruit spots and anthracnose diseases, e.g., of beans, clovers, cucurbits, citrus, banana, potato. Known hosts, in diseased condition, provide the best likelihood of successful collection: *C. lindemuthianum* (= *C. gloeosporioides*) on beans throughout North America, and *C. lagenarium* (= *C. orbiculare*) on cucumber. According to the literature, well-organized acervuli are produced on potato glucose agar, but isolates become heavily mycelial unless frequent (1-2 weeks) transfers are made using only conidia as transfer inoculum. Perfect states, when known, are in *Glomerella*. There are about 200 published names in the genus; only 11 are recognized by von Arx (1957). (Tiffany and Gilman, 1954; Sutton, 1962)

Diplodia

Cosmopolitan; saprophytic or parasitic on a great number of hosts (600-700 names in the literature). Two species are fairly common in North America on corn (*D. zeae* = *D. maydis*; and *D. macrospora*), both causing rots of ears and stalks; to be found on moldy ears and around nodes of grasses. A common saprophyte and parasite in tropics and subtropics is *Botryodiplodia theobromae* (syn. *Diplodia natalensis*). These are imperfect states of *Physalospora, Tryblidiella, Otthia,* and probably other genera. Example: *D. zeae* (= *D. maydis*); culture one week on hay infusion agar, by which time pycnidia are sparse but well-formed; after 2-3 weeks, there are good pycnidia with excellent conidia. (Zambettakis, 1955; Booth, 1958; Sutton, 1964)

Epicoccum

Cosmopolitan; saprophytic, perhaps weakly parasitic; common on many different plants (particularly in heads of cereal and grasses in wet weather), on decaying herbaceous debris of all sorts, decaying wood, and commonly isolated from the soil and the air. One species, *E. andropogonis* (syn. *Cerebella andropogonis*), is common on the "honeydew" *Sphacelia*-state of *Claviceps* on grasses, where it inhibits the formation of sclerotia and has the gross appearance of a smut. Isolates grow well in culture on a wide variety of media, but sporulation is erratic; early fluffy aerial mycelium declines in favor of scattered sporodochia as the cultures age (2-3 weeks); low nutrient media (hay infusion agar) is best, or sterilized straw or other herbaceous stems on surface of noncarbon agar. Most isolates require light exposure (50 ft-c or more for several hours) to initiate sporulation. Example: *E. nigrum*; culture 10-12 days on hay infusion agar to show lateral swellings of hyphae just beginning; sporulation is pronounced after 2-3 weeks. (Schol-Schwarz, 1959)

Fusarium

Cosmopolitan; includes saprophytes and many important plant parasites; commonly isolated from soil and plant debris and specifically associated with certain rots (foot rots of cereals, basal rot of *Narcissus*, dry rot of potato tubers) and wilts (of tomato, aster, potato, banana, cotton). Isolates are notoriously unstable in culture; sporodochial character often gives way in culture to scattered conidiophores; production of macroconidia may be suppressed in favor of microconidial production (and thus resemble *Cephalosporium*); pigment production is common. Some species are conidial states of Hypocreales, e.g., *Nectria, Gibberella, Calonectria, et al.* Examples: *F. decemcellulare*; culture 1 week on hay infusion agar to show macro- and microconidia; retains sporodochial character in culture; *F. moniliforme*. (Wollenweber and Reinking, 1935; Snyder and Hansen, 1940, 1941, 1945; Gordon, 1944-1959; Booth, 1959, 1960, 1971b)

Geotrichum

Ubiquitous in soil and dairy products; saprophytic, sometimes pathogenic in human respiratory and gastrointestinal tracts; may well appear in dilution plates prepared from soil, unpasteurized milk, cow dung. Example: *G. flavobrunneum*; sporulates well after about 14 days on hay infusion agar; grows more rapidly on malt agar, but the method of sporulation shows less well. (Carmichael, 1957; Miller *et al.*, 1957)

Gliocladium

Widespread; commonly saprophytic in the soil and on plant debris, saprophytic and parasitic on other fungi. Usually grows rapidly and sporulates in culture on several media. Conidiophores are usually in relatively dense clusters, as in *Penicillium*, but occasionally (particularly in young growth) are isolated in whorls, as in *Verticillium*. Perfect states, when known, in *Nectria*. Example: *G. deliquescens*; culture two days on hay infusion agar. (Booth, 1959; Hanlin, 1961a; Barnett and Lilly, 1962)

Humicola

Widespread in temperate and tropical regions; some species can be isolated from soil, wood, and other decaying cellulosic materials, others from hay (thermophilic), and one from marine habitat on wood. Some species produce a simple phialosporic state in association with the aleuriospores. Large aleuriospores tend to collapse in slide mounts; mount in lacto-phenol, heat gently. Example: *H. grisea*; culture 10 days on potato glucose agar. (White and Downing, 1953; Bunce, 1961)

Monilia

Widespread, notably as parasites of Rosaceae, Cornaceae, and Ericaceae; commonly develops on overwintered sclerotized fruits, but also on leaves, immature fruit, and twigs. Can be found on sclerotized fruits that have a powdery coating, or developed from fruits in a moist chamber. Conidial states also produced in *Neurospora*. Example: *Monilia* sp. (conidial state of *Monilinia fructicola*); shows good growth and sporulation after 5 days on potato glucose agar. Microconidial state also develops well as clusters of phialides sessile on hyphae; morphologically identical with *Botryophialophora*. The form-genus name is applied ambiguously to more than one group of fungi; it is used here for the macroconidial state of cup-fungi of the genus *Monilinia* (a segregate from *Sclerotinia*). (Walter, 1969; Barnett and Hunter, 1972)

Oidiodendron

Probably widespread in temperate climates (but reported at least once from tropics); can be isolated from soil (primarily peat and bogs) or from decaying wood and plant debris. Growth in culture is relatively slow. Example: *O. griseum*; sporulates after 10 days on potato glucose or malt extract agar. (Barron, 1961, 1962)

Pestalotia

Cosmopolitan; parasitic or saprophytic on a wide range of plants; commonly found on leaves but also on canes, twigs, tubers; promising sources are leaf spots of oak, rhododendron, rose, pine, eucalyptus, maple. Many of the species grow and sporulate readily in culture on ordinary media. Perfect states have been reported in *Pestalopezia, Leptosphaeria, Broomella*. Example: *P. virgatula*; good surface and submerged acervuli appear on potato carrot agar after 1 week. (Steyaert, 1949; Guba, 1961; Sutton, 1961; Shoemaker and Müller, 1963)

Phialophora

Widespread; saprophytic on plant debris, wood, and blue-discolored wood pulp and in soil; parasitic, including a disease of carnation and chromoblastomycosis in man. Shows slow to moderate growth in culture. Example:

P. verrucosa; restricted growth but excellent sporulation after 5 days on hay infusion or potato carrot agar. (Hughes, 1951; Cain, 1952; Schol-Schwarz, 1970)

Phoma

Cosmopolitan; reported as saprophytic or parasitic on any part of any living or dead plant; some species related to specific diseases of commercial crops (beet, citrus, turnip and other crucifers); frequently isolated from soil, plant debris, painted surfaces, paper products, cellulosic fabrics. Many isolates grow rapidly and fruit well in culture. There are reports of perfect states in *Pleospora*, *Leptosphaeria*, *Mycosphaerella*. (Morphologically similar species occurring on leaves customarily are classified in *Phyllosticta*.) Example: *P. pigmentivora* (= *P. herbarum*); grows well on potato carrot agar, one week. (Grove, 1935; Boerema, 1964; Walker, 1969)

Sclerotium

Cosmopolitan; parasitic, commonly on underground plant parts but also at and just above soil line; includes the omnivorous *S. rolfsii* (crop plant hosts include tomato, tobacco, olive, apple, cereals, legumes) and other species more or less restricted to such hosts as onion, rice, tulip and gladiolus. Sclerotia should be sought in basal rotted stalk tissues of corn, sorghum, cotton, soybean and other legumes, tomato, tobacco, melons, gourds. Isolate from inner tissue of surface-sterilized sclerotia. Some species are imperfect states of Discomycetes and Hymenomycetes (e.g., *S. rolfsii* = *Pellicularia rolfsii*). Example: *S. rolfsii*; few, typically scattered sclerotia formed after 2-4 weeks on potato carrot agar. (West, 1947; Walker, 1969)

Scopulariopsis

Cosmopolitan; very common saprophyte in soil, on semi-dry plant materials in late stages of decay (grains, vegetation), and on animal products relatively rich in nitrogen (leather, wool, cheese); occasionally isolated from skin and nail infections, but pathogenicity is problematic (i.e., secondary invaders). Several species are imperfect states of *Microascus*. Example: *S. brevicaulis*; culture 1 week on hay infusion or potato glucose agar. (Morton and Smith, 1963)

Trichothecium

Cosmopolitan; saprophytic on wood and herbaceous debris, dung, fabrics, paper; parasitic, causing pink rot of apples. Cultures readily on some ordinary media, but potato glucose agar is not recommended. Example: *T. roseum*; culture 4 days on hay infusion agar. (Rifai and Cooke, 1966; Kendrick and Cole, 1969)

Trichurus

Probably widespread; saprophytic in plant debris and as isolates from soil; grows well in culture. Example: *T. spiralis*; all stages show well after 1 week on hay infusion agar. (Morris, 1963; Barron, 1968)

ASPERGILLUS AND *PENICILLIUM*

The genera *Aspergillus* and *Penicillium* probably represent the most abundant and the most commonly encountered of all fungi. Both are world-wide in distribution and occur in the air and in the soil from the tropics to the arctic. They occur on all types of vegetation undergoing decomposition. The aspergilli, in particular, are capable of utilizing an enormous variety of substrates, and it is difficult to find a substance containing organic matter and a little water (sometimes very little) on which they cannot grow. In the tropics they colonize everything.

Many of the most active producers, decomposers, converters and pathogens produce no known perfect state*, hence tend to be ignored; while those species that have a sexual stage often receive minimal attention in the classroom. Such treatment in no way reflects the prevalence or importance of these molds. Hundreds of species have been described in each of these genera, and much duplication of description under different names has occurred. Even so, Raper and Fennell (1965) recognized 132 species and 18 varieties of *Aspergillus* as valid and identifiable; while Raper and Thom (1949) recognized 138 species and 4 varieties of *Penicillium*.

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*See p. 149 for discussion of perfect states in Eurotiaceae.

Isolation

Certain species of *Penicillium* and many species of *Aspergillus* may be isolated by direct transfer to suitable laboratory media with a finely pointed needle from such natural habitats as fruit, cheese, textiles, spoiled grain, moldy plant material. All of the penicillia and most of the aspergilli grow well on malt extract agar (M-48) and, because of the diverse nutrients it contains and its acid pH, it is generally recommended as a primary laboratory substrate. After the purity of the culture has been established beyond question, recultivation on Czapek's solution agar (M-89) is desirable since these fungi, as a rule, develop their most characteristic colony patterns and pigmentation on this medium.

The great majority of species, however, cannot be isolated by direct transfer, and some type of dilution plating is required. This may be done by streaking a dilute suspension of soil or other material on a nutrient-poor medium, such as dilute hay infusion (M-12) or soil extract agar (M-27), allowing the fungi to develop miniature colonies, and then reisolating selected types by needle transfer. A second method is to make a series of dilution platings of the sample. For most soils dilutions of 1:100 to 1:10,000 are generally satisfactory. Here again, some nutrient-poor medium should be employed; otherwise species of *Trichoderma*, mucoraceous fungi, and other rapidly growing forms will quickly obscure whatever colonies of *Aspergillus* or *Penicillium* are present.

Warcup's technique (Warcup and Baker, 1963) is especially useful for isolating many ascosporic species. The sample of soil is soaked in 60% ethanol for 5-10 min to reduce the number of bacteria and to destroy most of the fungi that are present in an asexual form. Small bits of soil are then removed and placed in sterile Petri dishes to which is added molten Czapek's solution agar, enriched with 0.05% yeast extract to accelerate growth and sporulation and adjusted to pH 4.0 with phosphoric acid to limit bacterial growth, or, perhaps better, potato carrot agar (M-17), which may well enhance ascocarp formation by Pyrenomycetes and small Discomycetes.

Cultivation and Examination

Colonies grown on agar media in Petri dishes are most useful for observation and comparison. When these are inoculated at three equidistant points, maturation proceeds most rapidly at the interfaces and fully developed conidial structures may be observed there while young and developing fructifications are to be found near the growing margins. Of several media that may be usefully employed, Czapek's solution agar is generally the most diagnostic, particularly with reference to colony patterns, texture, exudate and pigmentation. Malt extract agar, as a rule, supports more rapid growth and sporulation, and usually provides the best source of conidial and ascosporic structures for microscopic mounts. Many of the aspergilli are osmophilic, particularly members of the *A. glaucus* group, and for these Czapek's agar enriched with 20% or more sucrose provides optimal growth and development. For certain species, e.g., *Penicillium chrysogenum*, Czapek's agar enriched with 1% corn steep liquor is especially useful for enhancing growth, pigmentation, and the production of colony exudate. Hay infusion agar (M-12) is most useful in making isolations from soil and other natural substrates; it is often beneficial also for obtaining sporulation in species and strains that tend to be predominantly mycelial on the substrates of higher nutrient content.

With few exceptions, the aspergilli and penicillia grow optimally and sporulate well when incubated at 24-26°C. Light is rarely required for sporulation, although in types with long conidiophores it may induce a phototropic response. Relative humidity is not critical within a reasonable range.

Colonies suitable for comparative observations develop within 7-10 days in most species of *Aspergillus* and *Penicillium*, and mounts of conidial structures for microscopic study may be made from cultures of this age. In species that possess a sexual stage, ascocarp initials may be evident near the colony margins at this time, or their appearance may be somewhat delayed; mature cleistothecia with ripe ascospores are usually present after about three weeks, but these may appear earlier in some cases and very much later in others.

Colonies should be first examined with a low-power dissecting microscope. With a fine needle, remove a very small fragment of basal mycelium bearing young but sporulating conidial heads (or ascocarps of differing age, if applicable), place the fragment in a drop of 70% alcohol (or Tween 80) on a clean glass slide to wet the specimen and to remove air bubbles, tease apart gently, refloat in a small drop of lactophenol mounting fluid, and cover with a clean thin cover slip. A small amount of cotton blue (Poirrier's blue) may be added to the mounting fluid if staining is desired.

Conservation

Species of *Aspergillus* and *Penicillium* may be maintained successfully by periodic transfer to fresh agar slants of Czapek's solution or malt extract agar, with the exception of the *A. glaucus* group and other osmophilic species of *Aspergillus*, which should be cultivated on Czapek's agar with 20% sucrose. After cultures are allowed to sporulate, they can then be stored in a refrigerator at ca. 4°C for up to eight months without loss of viability.

Species of both genera may be stored in soil and thus extend their viability appreciably. Cultures grown on agar are allowed to sporulate, conidia or ascospores are suspended in sterile water, and enough of this suspension is added to tubes of sterilized garden loam to moisten the soil. The preparations are then allowed to dry at room temperature and stored in the refrigerator. A single soil culture, if handled carefully, may provide viable material for several years.

For long-term storage, lyophilization is strongly recommended. Species of *Aspergillus* and *Penicillium* so preserved have been found viable after more than 20 years. For instructional purposes a large number of lyophilized preparations may be processed initially and one of these opened each year to provide material for class study.

Representative Species of Aspergillus

The uncertainty of isolating a particular species from a selected soil is such that use of verified strains, available from large culture collections throughout the world, is recommended to make classroom study and comparison more meaningful. The species cited below have been selected to demonstrate specific characters of major importance in the classification of the aspergilli. Primary separation into groups is based on the color, shape, and size of the entire conidium-bearing apparatus (conidial head). Group differences based on macroscopic characters of the conidial heads are easily observable, the pattern of the conidial head being determined in large part by the size and shape of the *vesicle* that is formed by expansion of the conidiophore apex. For separating intermediate groups, the shape, arrangement, comparative dimensions, and sometimes color of certain of the structural elements comprising the conidial heads are used as additional characters. These details must be determined by study, under high magnifications of a compound microscope, of cultures inoculated on Czapek's solution and malt extract agars and incubated at 24-26°C for a week to 10 days. These microscopic details are essential to all species comparison and identification work in conjunction with modern literature on the genus (Raper and Fennell, 1965).

Representatives of ten groups are suggested as an introduction to the genus.

A. clavatus Conidial heads large, long, clavate, blue-green.

A. fumigatus Conidial heads compactly columnar, gray-green, sterigmata uniseriate, conidiophores hyaline to greenish.

A. nidulans Conidial heads columnar, green, sterigmata biseriate, conidiophores reddish brown. Produces cleistothecia accompanied by globose hülle cells and containing red ascospores.

A. terreus Conidial heads compactly columnar, pinkish tan to cinnamon, conidiophores hyaline.

A. flavipes Conidial heads columnar, sterigmata columnar, conidiophores brown.

A. niger Conidial heads large, globose to radiate, black or brownish black.

A. versicolor Conidial heads small, radiate, dark yellowish green. Strains of this species may vary in head color from green to almost flesh pink, hence the species epithet. Globose hülle cells produced in conspicuous yellow clumps.

A. ustus Conidial heads small, radiate, dull greenish gray becoming brown in age, conidiophores red-brown. Irregularly twisted hülle cells produced in white clumps.

A. flavus Conidial heads large, radiate, yellow-green.

A. candidus Conidial heads large, globose to radiate, white, in age sometimes splitting into two or more divergent columns. Conidiophores smooth (in contrast to conspicuous roughness in white mutants of *A. flavus*).

Development in Aspergillus

Various species of *Aspergillus* provide excellent material for demonstrating the effect of culture medium, light, and temperature on development.

Effect of Medium On Czapek's solution agar, growth of *Aspergillus repens* will be thin and very limited in extent; on Czapek's plus 20% sucrose, growth will be rapid, widely spreading and cleistotheica will be produced in abundance. Members of the *A. glaucus* group are extremely osmophilic; one species (*A. halophilicus*) requires as much as 60-70% sucrose or salt for growth.

Effect of Light and Medium *Aspergillus ornatus* should be incubated on Czapek's solution and malt extract agars for two weeks at 24-26°C in the light and in complete darkness. On both media growth in the light will be predominantly or entirely conidial--very limited on Czapek but luxuriant and extensive on malt. In the dark, abundant cleistothecia and few or no conidial heads will be produced on malt agar; growth will still be limited on Czapek.

Effect of Temperature *Aspergillus janus* should be incubated on Czapek agar for two weeks. At 18°C the conidial heads are white and long-stalked; vesicles are clavate, conidia elliptical and smooth. At 25°C both green and white conidial heads are produced; limited masses of globose hülle cells may be seen. At 30°C conidial heads are green and short-stalked; vesicles are subglobose; conidia are globose and echinulate; conspicuous masses of hülle cells are usually present.

Phototropic Response Conidiophores of *Aspergillus giganteus* will show conspicuous orientation toward the light if incubated for 10 days in a 1:1 mixture of Czapek solution and malt extract agars in a container with a nonreflecting inner surface and a narrow slit on one side to admit light.

Representative Species of Penicillium

Primary separation into sections within the genus is based on the pattern and relative complexity of the conidial apparatus. Further separations depend upon the surface characteristics of the colonies as they develop on laboratory media, particularly Czapek's solution agar. Series, or groups of related species, within these larger subdivisions derive from such characteristics as the presence or absence of cleistothecia, the presence or absence of sclerotia, the pigmentation of the conidia *en masse*, etc. (Raper and Thom, 1949)

The following species of *Penicillium* are recommended for class study because they illustrate the three structurally different types of penicilli upon which major sections within the genus are based, and because they also show differences in colony pattern and development that are characteristic of important subsections and series. Many of the recommended species can be found readily in fruit and cheese markets and in soil.

Monoverticillata

Penicillus simple, consisting of a single cluster or verticil of bottle-shaped sterigmata borne at the apex of the conidiophore.

Penicillium javanicum--ascosporic, with parenchymatous cleistothecia; fairly abundant in soils, particularly those of tropics and subtropics.

Penicillium sclerotiorum--sclerotial and strongly pigmented; in soils, apparently rare.

Penicillium frequentans--very common in soil and on decaying vegetation; neither cleistothecial nor sclerotial in culture.

Asymmetrica

Penicilli varying in size and complexity, consisting of two or more levels of cellular elements, sterigmata, metulae, and branches so arranged that the structures appear irregular or one-sided. Sterigmata usually bottle-form.

Velutina Colony surface plane or velvety.

Penicillium chrysogenum--common in soil and elsewhere; penicillin producer.

Penicillium digitatum--causes "olive green" rot of citrus fruit. Thiamine deficient; poor growth, or none, on Czapek's solution agar; malt extract agar is to be preferred.

Penicillium roqueforti--used in production of Roquefort-type cheeses.

Lanata Colony deeply flocculent or lanose.

Penicillium camemberti and *P. caseicolum*--used in production of Camembert-type cheeses.

Fasciculata Colony surface appearing tufted or fasciculate due to the clustered arrangement of conidial structures.

Penicillium italicum--causes "blue green" rot of citrus fruit.

Penicillium expansum--causes "blue mold" rot of pomaceous fruit.

Penicillium claviforme--a strongly coremiform species.

Penicillium isariiforme--produces strongly phototropic columns of appressed hyphae several inches in length (on Czapek's solution agar in unidirectional light).

Biverticillata-Symmetrica

Penicillus biverticillate and symmetrical, consisting of a verticil of metulae, each of which bears a verticil of narrow, lanceolate sterigmata.

Penicillium purpurogenum--common in soil and on decaying vegetation; penicilli typical of the section.

Penicillium vermiculatum--ascosporic, with club-shaped ascogonia and loose cottony ascocarps.

17 Heterobasidiomycetes

JELLY FUNGI

Collecting

The following are needed for collecting in the field: specimen container, knife, sheets of newspaper, waxed paper bags, hand lens, searchlight or head lamp, insect repellent and appropriate clothing. There is no substitute for a first-hand knowledge of the fungi, but the recognition of specimens in the field may be greatly enhanced by consulting herbarium material, if available, and by the study of photographs and written descriptions.

For collecting jelly fungi, which are generally much smaller and less fragile than agarics, a roomy canvas bag or rucksack slung over the shoulder is suitable; others prefer a basket. The only item of clothing needing special attention is shoes, which should be of the heavy-duty type, or boots, when more appropriate to the terrain. A flashlight or head lamp helps illuminate parts of a log that are in deep shadow and sometimes enables the collector to locate specimens that might otherwise be left behind. This approach is especially to be recommended when one is searching for inconspicuous resupinate species.

The ecology of tremellaceous fungi has not been adequately studied, but some certainly appear to prefer one substrate over another. *Pseudohydnum*, *Phlogiotis*, and some species of *Dacrymyces* have been collected predominantly from coniferous wood, whereas there are species of *Exidia*, *Tremella*, and *Auricularia* that have been found only on hardwoods. There are also important differences as between temperate and tropical species. Any wooded area should be considered potentially good collecting ground, but it is important to work systematically, turning over logs and inspecting them with care.

Most jelly fungi will be found growing as saprobes on woody substrates. When possible, logs should be rolled over and the under surface carefully examined. These fungi (and others too, of course) may be found in pockets or irregularities that retain moisture. Many of them are, as the name implies, gelatinous, but appear so only when wet. When dry, even the most gelatinous members become quite tough, horny or brittle. Among the genera in this category are most species of *Auricularia*, *Calocera*, *Dacrymyces*, *Exidia*, *Phlogiotis*, *Pseudohydnum*, and *Tremella*. Some tend to be waxy or waxy-gelatinous when wet, as are many species of *Sebacina* and *Gloeotulasnella*, while others remain arid, such as members of the genera *Septobasidium*, *Phleogena*, *Heterochaete*. and *Eichleriella*.

If collections are made shortly after a rain, some gelatinous basidiocarps are to be expected, most of which increase appreciably in size upon imbibing water. For example, dry *Exidia glandulosa* often appears as trivial, black, irregular patches on a dead branch and may even be missed by the inexperienced collector. But when soaked, the fruiting bodies expand greatly, are highly gelatinous, and evidence conspicuous brownish lobes.

Some species, notably resupinate ones like *Sebacina* spp., *Heterochaete* spp. and *Tulasnella* spp., are extremely inconspicuous or virtually invisible to the naked eye. If wet, they may be spotted with a hand lens as irregular, greyish or nondescript (occasionally more brightly colored) smears on wood. If dry, they are easily missed. The collector is advised to gather fallen twigs or branches suspected of bearing resupinates and, upon returning to the laboratory, to place them in moist chambers and observe under the binocular at intervals for 48 hours or longer.

Each collection should be wrapped in a separate sheet of newspaper together with a data slip on which is written the location, the date and brief notes on color, consistency, substrate, and other pertinent details. The packet is then secured with a rubber band. If specimens are to be studied upon return to the laboratory or soon thereafter, waxed paper or plastic bags are more effective than newspaper in maintaining collections in the fresh, expanded, gelatinous condition.

Laboratory Study

Specimens may be examined directly, placed in moist chambers, or preserved.

Fresh Material Jelly fungi may be prepared for microscopic examination by crushing or by free-hand sectioning. With a scalpel cut out a small fragment of the basidiocarp, including the hymenium. In many tremellaceous fungi the hymenium is amphigenous, that is, borne on the entire exposed surface; in others it is unilateral--when in doubt, cut from both surfaces. Mount in a drop of water on a clean slide. Replace the water with a drop or two of absolute alcohol; drain away and add a large drop of 3% KOH. Next add a drop of phloxine stain to the KOH, using a separate dropper to avoid contaminating the phloxine bottle with KOH. Add a clean cover slip and crush the specimen, using a glass rod or other suitable tool. Apply sufficient pressure by tapping the cover slip to flatten the preparation. Drain away any excess stain with absorbent paper and replace with KOH as needed. The fungus will appear pink to red, but the KOH should remain colorless.

For free hand sections the technique used above may be modified by cutting thin sections (10-15μ) of the fungus and by applying little or no pressure to the cover slip after staining. This method is indispensable in determining the structure of the basidiocarp. All that is needed are cylinders of elder pith and a sharp straight razor or a new single-edged razor blade. A cylinder of pith (about 5 cm long and 10-15 mm in diameter) is split in half on the long axis. The material to be sectioned is oriented as desired, between the halves, so that the exposed surface of the specimen lies flush with the top of the cylinder.

Moist Chambers Moist chambers are best made of deep, oversized Petri dishes or battery jars provided with glass covers, into which pieces of wood bearing fungi are placed for observation. Tremellaceous fungi, if present, may develop their characteristic gelatinous appearance within 24 hours, but it is best not to discard material for several days, examining it at intervals.

Spores and Spore Prints Spore prints of tremellaceous fungi have generally been neglected by collectors, but should be obtained whenever possible. This is most easily done with fresh material, the basidiocarp being placed, hymenium downward, on white paper (or on glass) for periods up to 24 hours. As the fruiting body dries (at room temperature), mature basidiospores will be shot away. Their color as seen *en masse* should then be recorded. Basidiospores obtained from spore prints often give more reliable measurements than do those taken directly from the basidiocarp, since the latter samples will usually have numerous immature spores, many of which are atypical in size and septation. Spore prints can also be obtained from basidiocarps that have been recently dried. These may be soaked and treated like fresh material. Of course there will be some disappointments, but revived specimens often yield a satisfactory crop of spores.

The basidiospores of Tremellales commonly develop secondary spores by an asexual process of repetition whereby mature spores produce a sterigma upon which a secondary and smaller spore is formed. This situation may complicate the interpretation of spores but must be taken into account.

Available species of the common and widely distributed genera *Exidia*, *Dacrymyces*, and *Auricularia* (fresh or dried) will suffice to show three different basidial types: the subsphaerical, cruciate-septate basidium of *Exidia* (exemplifying the family Tremellaceae); the two-pronged, unicellular basidium of *Dacrymyces* (Dacrymycetaceae); the elongated, transversely septate basidium of *Auricularia* (Auriculariaceae). Of these, the *Auricularia* basidium is somewhat more difficult to demonstrate in a crush mount because of the great tenacity of the gelatinous hyphae in the hymenium. It is therefore recommended that thin free-hand sections of the basidiocarp be cut first and that after the position of basidia in the hymenium (stained) is observed, the mount be crushed in order to separate individual basidia for study.

Spores germinate readily on a variety of agar media (malt agar may be used or plain agar to which about 5% glucose has been added), but basidiocarps are rarely formed. If a fragment of a freshly collected basidiocarp is attached by adhesive to the under surface of a Petri dish lid, basidiospores are likely to be projected onto a thinly poured agar surface that should then be inspected at intervals under the compound microscope for evidence of germination. Spores may then be removed to a slide for further examination.

Preservation

The best way of preserving tremellaceous fungi is by drying, by either natural or artificial heat. If specimens are laid out in the sun, the smaller ones should be dry within a few hours. Air-dried specimens may be used for subsequent culture work, since spores remain viable under these conditions. This is far less true of material dried by artificial heat. For routine preservation, collections should be placed in a dryer, which may be a simple box with a screened top and fitted with light bulbs or a more elaborate piece of apparatus especially designed for the purpose.

Drying should be neither too rapid, too prolonged, nor at too high a temperature, since the material may otherwise undergo deleterious structural changes. If a closed cabinet equipped with removable screens is used, care should be taken to allow for proper circulation of air through a screened top. An electric hot plate set at low heat on the floor of the cabinet generally satisfies the conditions needed. Properly dried specimens are suitable for filing in the herbarium for future use.

See also Coker, 1920; Lowy, 1952, 1955; Martin, 1952.

SEPTOBASIDIUM

Species of the genus *Septobasidium* (Couch, 1931, 1938) are world-wide, occurring on the limbs and, less frequently, on the leaves of living plants, and are always symbiotically associated with scale insects. The fungus furnishes a shelter and protection for the insects, while the insects supply nourishment and a means of distribution for the fungus, this partnership thriving at the expense of the tree. Most of the species of this genus are tropical, but some are common in the temperate zones. A good one for study in the Eastern United States is *Septobasidium burtii*, reported from Long Island, N.Y., to Northern Mexico. It is most frequently found on *Quercus*, producing on the smaller limbs and branches a circular, corrugated, brownish, perennial growth. Although the fungus-insect relationship can be observed throughout the year, the best time for study is in mid-spring, after the first warm rains have stimulated the reproduction activities of both fungus and scale insects.

Fresh material will show a gross structure of more or less radial ridges and valleys. Here and there are broad openings in the top surface of the fungus, each opening partly covered by a thin flap of fungal tissue. In many specimens one can detect concentric rings of growth, a new ring forming each year. Under a low-power binocular dissecting microscope, the fungus will be seen to consist of three regions: (1) the white subiculum closely pressed to the bark; (2) the radial ridges arising from the subiculum; and (3) the top horizontal layer arising from the ridges. Between the ridges are anastomosing tunnels that widen here and there to form spacious chambers. The chambers open to the outside through the openings noted earlier. Beneath the fungus are two kinds of scale insects: healthy, nonparasitized ones and parasitized ones. Insert a needle through the door in the roof and lift the roof up to expose the yellowish, plump, healthy female in her chamber, protected by the fungus but not in contact with it. The parasitized insects are embedded in the fungus, are much smaller than the healthy ones, and are difficult to find. Coiled haustoria should be evident in the coelom, but not in contact with any vital organs. To see these better, open the insect with dissecting needles. It is through the living but parasitized insects that the fungus is nourished.

Reproduction of the Fungus

The probasidia are formed during the winter months and germinate to form basidia when the warm spring rains come in mid-April. Basidial formation tapers off from this time on and ceases entirely before midsummer. A vertical section through the hymenium in mid-April will show sterile hyphae, probasidia, basidia, and basidiospores. The basidia are cylindrical and at maturity thrice septate and thus four-celled, each cell forming a short sterigma and one basidiospore.

Reproduction of the Scale Insects

The scale insect associated with *S. burtii* on *Quercus palustris* is *Aspidiotus osbornii*. In mid-April the healthy overwintering females beneath the fungus are loaded with eggs ready to hatch, a process stimulated by warm weather. During sporulation of the fungus, the nymphs that crawl over the spore surface accidentally pick up the spores and become infected. Infection stages may be found by collecting the crawling nymphs two to three hours after a rain, mounting them on a slide in a drop of water under a cover slip and examining for spores attached at the bases of setae and on the antennae and legs. It is by the infected nymphs that the fungus is distributed.

SHADOW YEASTS

Species of *Sporobolomyces* occur commonly on leaves, stems, fruits, seeds, and similar materials from higher or lower plants, particularly on material that is old and in the process of dying or decomposing. For class use, collect representative samples of cereal grasses, dead and decomposing leaves, wild flowers, parts of mosses or ferns, and similar items. Samples collected in a relatively cool climate and those that are moderately moist, e.g., a few days after a rainstorm, are most suitable. These can be stored in a refrigerator for not longer than a week, but should not be dried.

Isolation

Isolation is based on the forcible discharge of the ballistospores from cells present on the experimental material, and trapping the discharged spores on a suitable medium upon which they can grow and develop microcolonies. The colonies are purified by inverting the initial plate over a second plate and allowing the primary colonies to discharge spores onto the second plate.

Three or four large Petri dishes are required, each poured with cornmeal agar (M-6); neopeptone-glucose agar, or any agars used in yeast studies, can also be used here. Large Petri dishes allow one to use a greater quantity of plant materials, but if they are unavailable, ordinary Petri dishes can serve the purpose. The plant materials are attached with Scotch tape to the inside of the Petri dish cover. An alternative method is to place a coarse flexible metal screen over the bottom half of the Petri dish, bending the metal at the edge in two or three places to keep the screen in place. Deposit the plant material on the screen and put the cover in place. Incubate at room temperature or, preferably, at 18-20°C. Observe periodically for the formation of tiny pinkish colonies beneath the screen on the cornmeal agar. If the material contains *Sporobolomyces* cells, colonies may be observed after about four days. Members of *Bullera* and *Sporidiobolus* can be isolated by the same method.

As soon as colonies are observed, the bottom of the plate is placed upside down for one hour on another Petri dish bottom that also contains cornmeal agar. The sides are taped with Scotch tape. Cells of the initial colonies will discharge spores during this hour onto the medium in the bottom dish. The cover is then replaced on the second dish and the plate incubated for three days to allow the discharged spores to form colonies.

This process of purification can be repeated, or an isolated colony can be suspended in a small amount of sterile water in a test tube and the suspension restreaked by conventional purification procedure. Finally, a purified colony is transferred to a slant of malt extract agar stock medium. During isolation, cornmeal is preferred over malt extract agar because cultures of *Sporobolomyces* shoot more spores on a relatively poor medium such as cornmeal extract, and contaminating fungi show less tendency to spread rapidly on this medium.

Laboratory Study

From a pure culture make an inoculum in the form of letters on malt extract or cornmeal agar in a Petri dish. Place this inoculated plate in an inverted position over the bottom of another Petri dish containing the same medium, and tape the sides with Scotch tape. After incubation for three days, the letters will appear as mirror images on the bottom dish, due to growth and spore discharge from the top dish.

Microscopic examination of young cultures will show budding cells, pointed sterigmata, and asymmetrical, somewhat crescent- or kidney-shaped ballistospores. In some cases the spores may still be attached to the sterigmata, but the best way to see attached spores is to inoculate *Sporobolomyces* on a thin layer of cornmeal agar on a slide. After several days, place the slide directly under the microscope and observe the aerial sterigmata with attached spores.

See also Lund, 1956; do Carmo-Sousa and Phaff, 1962.

RUSTS

Although *in vitro* culture of rusts is impractical for class use, they are extremely common in all terrestrial habitats and readily obtainable for class use; some common species can be readily maintained on their hosts and can be kept indefinitely as dried specimens without troublesome changes in morphology.

Collecting

Rusts can be found wherever phanerogams occur, from deserts to marshes to mossy rainforests. Because they are highly host-specific, rusts are limited by the distribution of their hosts. Such open areas as roadsides, fields, edges of forests, meadows, and fence-rows are more productive than forests, because most rusts occur on herbaceous plants. In dry regions, shaded banks, where dew remains longest, will have more rust than will areas that are quickly dried by early morning sun.

Rust infections have various symptoms that are more or less readily distinguished from symptoms incited by other parasitic fungi. The experienced collector can distinguish these with the naked eye, but the beginner will need a hand lens. Aecia, uredia, and telia are generally borne on the underside of the leaf. The upper side shows pale or discolored spots not much larger than the diameter of the sorus. Usually it is necessary to turn the leaf over to be certain of the situation.

Several forms of aecia are found among the rusts, the commonest being the aecidioid (cup-shaped) form, characteristic of most species of *Puccinia* and *Uromyces*. Several to many aecia occur in a tight, circular cluster 2-20 mm in diameter, the size of the cluster depending upon the species. The cup-shape form of each aecium is usually just barely discernible to the naked eye. Spermogonia are smaller and not always visible. They may appear as tiny dark specks in the center of the yellow or reddish spot on the upper side of the leaf above the cluster of aecia. Aecia borne on a systemic mycelium are readily detected because the infected stems are always erect, even if the host is normally prostrate. The leaves then are uniformly and densely covered with aecia.

Uredia are almost always scattered over the leaf. They are found mostly on the underside of the leaf, but are often on both sides of grass leaves. Uredia vary in shape and color, according to species. On broad leaves they are more or less round, but on grass leaves they are often elongated. Usually they have the form of blisters, up to 1 mm wide, because the spores push the epidermis up before breaking through it. Sometimes a ring of uredia forms around an old uredium, resulting in a cluster. The spores, in mass, are usually some shade of brown to orange, dull to bright, depending upon the density of the pigment in the spore wall, which in turn depends on the species. The brightest spores are without pigment in the wall, and the orange protoplasm loses its color soon after death, eventually rendering the spores white in mass.

Telia of macrocyclic rusts often occur along with uredia in late summer. They occur first on leaves that are beginning to die, and they are almost always dark brown or blackish. Telia may be round or elongated and usually break through the epidermis. They are not as powdery as uredia because the teliospores usually remain attached to the sorus. Telia are about the same size as uredia or may be slightly larger. Teliospores may form within old uredia.

Telia of microcyclic rusts usually occur in tight clusters 1-20 mm wide, mostly on herbaceous dicots. Old clusters appear grayish in the center because of the mass of hyaline basidia emerging from germinated teliospores. To find clustered telia without uredia is a good indication that the rust is microcyclic, certainly whenever spermogonia are present.

Larvae of certain tiny flies (*Mycodiplosis*) feed upon rust spores. The presence of these tiny pink to orange larvae, up to 1 mm long, serves as an additional field "symptom" because they are almost always present.

The most convenient collecting containers are polyethylene bags, preferably without holes; they must be washed well before re-use. Collected plants will not wilt if the bag is kept closed, but the bag must be kept shaded to avoid overheating. One's thumbnail is the most convenient tool for pinching off leaves; a sharp knife is helpful in collecting tougher plant parts. If whole plants are to be transplanted, they must not be allowed to wilt. It is best to carry water and to transplant into pots of soil if the collecting trip is to last several hours. Try to get most of the roots, with minimum disturbance, and remove some of the foliage to compensate roots that are lost.

The host plant must be identified at least to genus before one can identify the rust species. Therefore, it is well to collect enough of the host to facilitate its identification; whenever possible, include an inflorescence. If the host plant does not have needed diagnostic features at the time the rust is collected, note the location and return later to observe the plant until such time as it can be identified. At the same time, watch the development of the rust.

Note the exact location of the collection sites so that they can be visited several times during the season. Watch the development of the rust, especially to obtain the other stages of the cycle. It is entirely feasible to visit a given site every year, since autoecious rusts, and those heteroecious rusts that have both hosts intimately associated, should continue to be present.

Storage of Material

Collections that are to be dried for long-term preservation should be treated as phanerogams, i.e., dried in a plant press. An excellent substitute for a plant press is a phone directory or any such book made of absorbent paper. Spread the severed leaves flat, evenly spaced, so as to cover not over half of the page surface, and avoid overlapping the leaves. Label each layout and allow several pages between successive layouts. It is important that the plants become brittle-dry within three days, else they will discolor. Warming is not necessary if the atmosphere is dry. It is helpful to flip the pages every day to hasten drying. When the leaves are dry, they can be conveniently stored in envelopes or folded paper packets. Label each with the rust species, host, collection date and place, collector, and identifier. Specimens must be periodically fumigated with paradichlorobenzene to prevent destruction by insects.

Collections that are to be used while the spores are living must be handled with due regard to factors that affect viability. Avoid overheating; do not expose bagged plants to direct sunlight or place them in a hot automobile. Spores must be dried as soon as possible; do not leave the plants in the bag any longer than absolutely necessary. It is best to put rusted leaves directly into a desiccator or plant press at the collection site, or to transport the host plant unenclosed. At room temperature, spores remain viable for periods of a few weeks to several months only. Storage in a desiccator, in a refrigerator, will keep spores viable for a year or longer, depending upon the species and the spore type. Certain rusts keep even longer in a freezer. They can be kept for many years stored in vials in liquid nitrogen (Davis *et al.*, 1966).

Maintenance on Living Hosts

For greenhouse culture of rusts it is best to try only species that have a uredial stage (macrocyclic), such as the grass rusts. Corn rust, *Puccinia sorghi*, is especially easy to maintain and will form telia as the leaves die. Corn can be inoculated by placing a drop of water-suspended uredospores in the first whorl of young leaves of a seedling about 10 days after the seed is planted. As the leaves expand, the first uredia form near the leaf-tips in about six days. Frequent reinoculation yields more uredia. Soon there will be so much rusted foliage that squirting the plants when they are watered will spread the rust. About every month it is desirable to start new seedlings, which should be placed among the older plants. Telia on nearly-dead leaves are black and do not break through the epidermis. They can be overwintered outdoors and the spores germinated in the spring, although generally the microcyclic rusts are much more dependable for demonstrating germinating teliospores.

Microcyclic rusts can be maintained in the greenhouse if given periodic attention to assure new infections. Moist conditions must be provided for a long enough period to allow teliospore germination and for their basidiospores to have time to infect the host. This requires more time than is needed for infection from uredospores. Microcyclic teliospores form basidiospores in 4-12 hours, and infection probably occurs in 1-2 hours more. Wet the infected plants and cover them with a plastic bag. When the centers of the sori become gray, it will indicate that some teliospores have germinated.

When many basidiospores are present (microscopic check is necessary), transfer spores to moistened leaves and recover with plastic for several hours. It is important that the plant not be in direct sunlight while it is covered. A good microcyclic rust for greenhouse culture is the hollyhock rust, *Puccinia malvacearum*, on the common prostrate weed *Malva neglecta* or on one of its relatives. Another is the cocklebur rust, *P. xanthii* on *Xanthium* spp., but the host needs supplemental light during winter to keep it in the vegetative stage.

Many rusts that are difficult to culture in the greenhouse can be readily maintained in a backyard "rust garden." Any autoecious rust with a self-seeding or perennial host will maintain itself from year to year with little or no help. Many heteroecious rusts are easily maintained if both hosts can be grown in close proximity. Of course, the hosts must be compatible with the domestic habitat, preferably perennial or self-seeding, and the rust must be well established before the first winter. Periodically wetting the foliage and rubbing the leaves together will help increase the incidence of rust.

Laboratory Study

If the uredial stage is used as inoculum, it is easy to propagate the rust to epidemic proportions on a small scale in greenhouse or yard. By timing the production of spores from generation to generation and determining the approximate number of spores formed, one can calculate roughly the potential inoculum increase in a given time period. Having once related the density of uredia to gross effect on the host, one can show that an epidemic has the potential of occurring in a very short time after the introduction of the primary inoculum.

While morphological characteristics of rust can be demonstrated by either living or dried specimens, basidia are best demonstrated immediately after they germinate from teliospores, as they are especially susceptible to damage during processing into permanent preparations.

There are several good mounting media. Water can be used for fresh spores but dried spores are best mounted in lactic acid or other wetting fluid. Lactic acid is a good clearing agent, wets the spores, makes germ pores more visible, and resists evaporation. Other good clearing and wetting fluids are 3-5% chloral hydrate with a little glycerine added to retard evaporation, and 3% KOH. Stains are rarely used with rusts, but cotton-blue with lactophenol may be useful with fresh basidia. An excellent permanent mounting fluid is Hoyer's (SR-7) because it will receive fresh or wet material directly, without tedious dehydration, yet hardens in a few hours. It is even better than lactic acid for showing germ pores. Spores sometimes collapse initially in Hoyer's but return to normal in a few minutes. Trapped air bubbles are soon absorbed. The only disadvantage of Hoyer's is its incompatibility with commonly-used stains.

Spore Germination

Teliospores of microcyclic rusts germinate very readily as soon as they are mature. Teliospores of some species are most likely to germinate if they are left attached to the telium. The leaf, including the telium, should be moistened, and excess moisture blotted off. The whole leaf can be placed in a moist chamber or excised portions of telia may be placed on 2% water agar. The agar surface *must* be free of liquid water because normal basidia *will not* form if the spores are submerged in a film of water (Cunningham, 1966).

Teliospores of some demicyclic rusts germinate as readily as those of microcyclic rusts, and should be treated as above.

Teliospores of many, if not most, macrocyclic rusts must over-winter outdoors to break their dormancy. Some species respond to artificial alternate wetting and drying and freezing and thawing, but this is hardly worth the trouble if more responsive rusts are available. After allowing nature to take its course outside, one can periodically bring in samples to test. The spores could be ready to germinate any time after mid-winter. When the spores become dependably germinable, a quantity of spores can be stored in the refrigerator until needed.

Basidiospores are very sensitive to drying and germinate as soon as they are formed. Their viability period is very short--minutes to hours. In some rusts, such as *Gymnosporangium* and *Coleosporium*, basidiospores germinate repeatedly, each time forming a single secondary basidiospore on a sterigma.

Aeciospores of some rusts, such as *Gymnosporangium*, may require a short waiting period before they will germinate (Miller, 1939), but others may germinate immediately. Except for inoculation of plants, it is somewhat impractical to germinate aeciospores for class use. The addition of small amounts of sugars to the agar sometimes helps germination.

Uredospores usually germinate readily in one to several hours, depending upon temperature and species. The most dependable method is to place the spores on the surface of at least 2% water agar. The surface of the agar must be free from liquid moisture. Another method is to suspend spores in a drop of water on a slide, let the water evaporate to "fix" the spores to the slide, and then invert it over moist filter paper in a Petri dish. If a slight amount of moisture condenses on the spores, they will germinate. They must have free access to air. In small closed containers, such as Van Tieghem cells, germination may be inhibited.

Rusts for Class Use

The following rusts are so common and so widely distributed that at least some of them can be found in most areas. Certainly many additional rusts just as usable for class study or research tools will be abundant locally. See Arthur (1929, 1934) and Cummins (1959) for detailed descriptions of the rusts.

Coleosporium species are mostly heteroecious, spermogonia and aecia occurring on pine needles in spring and summer. Uredia and telia occur on various monocots and dicots, especially Compositae, in summer and autumn. Aecia are yellow to orange; telia are red to red-orange. Spores become colorless after death. Uredospores germinate within three hours on water agar. Teliospores germinate readily as soon as they are mature. They can be germinated on the leaf, or excised whole telia may be placed on water agar. Basidiospores form within 15 hours at room temperature. Infection of pine probably occurs in the fall, and mycelium overwinters in the pine needles to bear aecia in May and June. *Coleosporium* is very common by autumn; *C. asterum* is widely distributed on various Compositae, especially species of *Aster* and *Solidago*.

Cronartium species are heteroecious, producing conspicuous aecia on stems and cones of pines in spring. The gametophytic mycelium in stems is perennial, infecting large areas of twigs and branches. Infected stems can be forced to bear aecia earlier in the spring if they are brought inside and allowed to stand in water. Various dicots bear the uredia and telia. Teliospores germinate as soon as they are mature. *C. fusiforme* and *C. quercuum* occur on both two- and three-needle pines, and uredia and telia occur on *Quercus* spp. Telia are hair-like, and dense infection makes the leaf look coarsely pilose underneath. *C. ribicola* occurs on five-needle pines and *Ribes*.

Gymnoconia species are demicyclic and autoecious. The caeomoid aecia are golden-yellow or orange when fresh, borne on a systemic mycelium in spring and summer. The brown telia occur later and resemble those of *Puccinia*. *G. peckiana* is common on various species of *Rubus*. It has a common microcyclic derivative, *Kunkelia nitens*, that can be distinguished from the aecial stage of *G. peckiana* only by germination of the spores. The "aeciospores" of *Kunkelia* behave as teliospores, producing basidia and basidiospores.

Gymnosporangium species are heteroecious and mostly demicyclic. Spermogonia and conspicuous roestelioid aecia occur on various woody species of Rosaceae, in early to late summer. The peridium of each aecium is persistent, horn-like or fibrous. Aecia occur in clusters on leaves, fruits, or twigs, often on slightly hypertrophied tissue. Infected leaf tissue is usually yellow or orange on the upper side, making conspicuous spots up to 1 cm wide. The brown aeciospores often have to rest a short time before they will germinate (Miller, 1939). Infection of the primary host (various cedars, especially *Juniperus*) occurs in fall and winter. The mycelium in cedar bears its first telia the second April-May following infection, and, depending upon the rust species, the infection can bear telia from perennial mycelium every spring for many years. Telia can be forced to form earlier in the season if infected stems are brought inside and placed in water. Teliospores are borne on very long pedicels that make up macroscopic horn-shaped, strap-shaped, or mound-shaped telia capable of swelling several-fold when wet. Telia are most easily found when wet because they are then much larger and bright yellow or orange. Teliospores germinate readily in about three hours at room temperature as soon as they are mature, and basidiospores form within a total of about eight hours. A high percentage of the spores germinate during the first wetting, but some will wait for subsequent wettings. Meanwhile, the telia may be dried for later use. The telia are exhausted after several wettings. Individual telia may be divided into tiny portions without adversely affecting germination; thus a little collection can serve several classes. Red cedar, *J. virginiana*, is the most common telial host to many species of *Gymnosporangium*. *G. juniperi-virginianae* alternates with *Malus* spp.; the reniform cedar galls occur on green twigs and are not perennial. *G. globosum* alternates with *Malus* spp. and *Crataegus* spp.; the globose cedar galls are perennial on small twigs. *G. clavipes* alternates with *Amelanchier* spp., *Crataegus* spp., and *Malus* spp.; telia are borne on perennial cankers on branches of any size. *G. nidus-avis* alternates with *Amelanchier* spp.; the nonpersistent hemispheric telia are distributed evenly along green twigs, making conspicuous "witches brooms" caused by the systemic mycelium, or may be found in old stem cankers. Peterson (1967) describes several species common in western United States.

Melampsora has all of the life-cycle types. The orange to yellow caeomoid aecia occur in spring. Yellow uredia and brown to black telia occur on species of *Populus* and *Salix* and they alternate with species of *Larix*, *Abies*, or *Tsuga*. Most species on herbaceous hosts are autoecious. Flax rust, *M. lini*, also is macrocyclic. Teliospores must overwinter before they will germinate.

Phragmidium species have caeomoid aecia, all species are autoecious, and most are macrocyclic. All occur on Rosaceae, especially *Rosa, Rubus,* and *Potentilla.* Aecia are yellow to red-orange and occur on stems or leaves in spring. The upper side of the leaf spot is often red. Red-orange uredia occur on leaves from early summer onward. Black telia form on the leaves in autumn or sooner and must overwinter before they will germinate.

Puccinia and *Uromyces* make up most of the Uredinales and are so similar that they can be treated together. They differ only in the number of cells in the teliospore. Both genera contain species that exemplify all types of life cycle. One or more species have been recorded on most genera of monocots and advanced dicots.

Almost all grass and sedge rusts are heteroecious, and many of them alternate with dicots. Many grasses and sedges are host to several species (see Cummins, 1956). It is likely that one or two species can be found in any average suburban yard, on the lawn grass or on various weed grasses, through the summer and autumn. Uredia may be bright orange to rusty, depending upon the species. The black telia usually do not form until late in the season and usually need to overwinter before their spores will germinate. Grass rusts are easily maintained in the uredial stage in the greenhouse, but they do not persist from year to year outside except in warm climates, unless the alternate host is nearby. *Puccinia recondita* is one of the most common grass rusts encountered. Various forms of it collectively infect a total of several hundred grass species and many different alternate hosts. In moist woods, one can find aecia on *Impatiens* in spring and uredia and telia on *Hystrix* soon afterward. *P. recondita* also occurs commonly on quackgrass, *Agropyron repens*, and on the several cultivated grains, alternating with various species of the Ranunculaceae. *P. graminis* is common on cultivated and weed grasses, alternating with species of the Berberidaceae. *P. dioicae* and *P. caricina* are common on sedges. Their aecidioid aecia occur in spring on Compositae and Saxifragaceae, respectively. Their uredia are rusty of cinnamon-brown and the darker telia form during summer or later. Sedge rusts are commonly hyperparasitized by *Darluca.* The snapdragon rust, *P. antirrhini*, is apparently heteroecious but the alternate stage is unknown. Brown uredia and telia are common by late summer.

Most autoecious species occur on dicots; some are macrocyclic species. Every yard with dandelions, *Taraxacum officinale*, is apt to have *P. hieracii*. The primary and secondary uredia are brown. The dark-brown telia form in autumn. Infected plants are readily cultured in the greenhouse, but where winters are mild, material for class can be obtained from the yard the year round. Whenever sunflowers are grown, *P. helianthi* can be expected. The aecidioid aecia are not likely to be found unless volunteer seedlings grow near telia-bearing debris from the previous season. Brown uredia become abundant by summer, and black telia form in late summer, first on oldest leaves. *P. violae* is cosmopolitan on *Viola* spp. Its aecidioid aecia occur in early spring on hypertrophied spots on leaves and petioles. Brown uredia soon follow and dark brown telia occur by early summer. *P. menthae* occurs on various mints such as *Mentha* and *Monarda*. Aecidioid aecia occur in spring on stems and lower leaves, often causing hypertrophy. Cinnamon-brown uredia soon follow, and brown telia may be found by bloom time. *P. convolvuli* occurs on bindweed, *Convolvulus sepium*. The aecidioid aecia occur from spring to summer and are soon followed by brown uredia and black telia by mid-summer. Clover rust, *Uromyces trifolii*, is common where *Trifolium pratense* is abundant. The aecidioid aecia are not easily found unless one encounters a plant that had been heavily infected the previous season. The brown uredia become abundant by autumn and then brown telia form. *Uromyces euphorbiae* is common where *Euphorbia* spp. occur. Its systemic aecidioid aecia occur on erect seedlings in spring and early summer. The brown uredia soon become abundant and the darker telia can be found by mid-summer. This rust occurs faithfully every year once it is established. *U. polygoni-avicularis* occurs on the common prostrate knotweed, *Polygonum aviculare*. The aecidioid aecia occur in groups in spring, soon followed by rusty uredia. Some telia can be found by mid-summer, first on stems. The systemic aecidioid aecia of *U. ari-triphylli* can be found on the jack-in-the-pulpit, *Arisaema triphyllum*, in early spring even as the host leaves expand. The light cinnamon-brown uredia and light-chestnut brown telia occur in summer. A method for inducing systemic infection has not yet been worked out. *U. phaseoli* occurs on cultivated beans and other species of *Phaseolus*. It is readily cultured in the greenhouse.

As for autoecious demicyclic species of *Puccinia*, *P. mariae-wilsoniae* occurs on the spring-beauty, *Claytonia virginica*, wherever the host is common. The aecidioid aecia are conspicuous on reddish-orange hypertrophied leaves, petioles, or inflorescences, as the plants begin to bloom in very early spring. Brown telia soon follow. The rust and host must be watched frequently because the foliage dies only a few weeks after anthesis and then the rusted leaves cannot be found. *P. podophylli* is conspicuous on the mayapple, *Podophyllum peltatum*, wherever the host occurs. The large clusters of aecidioid aecia are reddish-orange and occur in early spring as the host leaves expand. A few dark-brown telia may occur above the aecia or at the base of the stalk then, but soon are common on the leaves underneath the angular yellow spots.

There are two especially common microcyclic species. *P. xanthii* occurs on cocklebur, *Xanthium* spp., and the giant-ragweed, *Ambrosia trifida*. The brown telia occur in large tight clusters, up to 1 cm wide, under yellow spots on the leaves. *P. malvacearum* occurs on hollyhock, *Althaea rosea*, and on mallow, *Malva* spp. The cinnamon-brown to brown telia are hemispheric and small.

Penetration of the Host

During the summer, collect sunflower leaves bearing uredial pustules, press and dry the leaves and store them in envelopes or plastic Petri dishes in the refrigerator. Urediospores of most rusts will remain viable for three or four months under such conditions (Bailey, 1923).

Inoculate the cotyledons of young sunflower plants grown from seed. The plants to be inoculated should be young enough so that the cotyledons are still green and in good condition. The cotyledons can be inoculated in either of two ways:

- Scrape uredospores from the dried sunflower leaves into 30 ml of water in a small beaker, strain through cheesecloth to remove leaf fragments, pour the resulting spore suspension into an atomizer and spray the cotyledons with the spore suspension.
- Spray the cotyledons to wet the epidermis thoroughly; then scrape uredospores from the dried leaves and apply them to the surface of the cotyledon with a scalpel.

The plant and pot can be enclosed in a plastic bag moist chamber and should be kept at a temperature near 20°C. The plastic bags can be removed 12-15 hours after inoculation. To observe formation of germ tubes and appressoria, penetration of the plant and formation of substomatal vesicles, remove strips of epidermis from inoculated cotyledons one, two, and three days after inoculation, stain the epidermal strips with aqueous eosin, and examine under the microscope.

Teliospore Germination

Spring Collect cedar-apple galls in April or May as soon as they show some extension of spore horns. Soak galls in water for about 30 min; then keep them in moist chambers for 4-12 hours. The time required for germination and for formation of basidiospores will vary according to the condition of the galls. A trial run, to determine correct timing, will be helpful. Optimum temperature for teliospore germination is reported to be approximately 24°C, but germination will occur over a fairly wide temperature range, and room temperature is satisfactory.

As soon as germination begins to occur, the material can be studied in several ways. Small pieces of the jelly-like spore horns can be examined microscopically so that various stages of teliospore germination can be observed. A spore print, composed of discharged basidiospores, can be obtained by leaving the gall on a glass slide in the moist chamber until an orange spore deposit is visible.

Fall Gather gall-bearing twigs at any time from November 1 to December 1. After cutting the twigs from the tree, place cut ends in water immediately. Keep twigs with cut ends in water until spore horns show signs of emerging (two to three weeks). Make a fresh cut at the base of the twig every three or four days, and change the water frequently so that bacteria or molds will not develop. As soon as spore horns begin to emerge, the material can be studied as described for spring.

Biology of Puccinia sporoboli

Materials Needed Overwintered telial material of *Puccinia sporoboli* on sand grass (*Calamovilfa longifolia*); seedlings of *Yucca* spp. in 4-inch pots; disposable plastic bags; atomizer; Petri dishes or similar containers that can be used as moist chambers; single-edge razor blades for making free-hand sections.

Procedure Overwintered telial material of *P. sporoboli* is usually ready to germinate by March or April. (If the class study is to be done in the fall, telial material can be frozen in ice or stored at 5-10°C until needed.) Beginning on March 1, check small samples of the material at two-week intervals by soaking ten 3-inch pieces of telia-bearing grass blades in water for a couple of hours, then placing the material in a moist chamber (such as a Petri dish lined with wet filter paper). The moist chamber should then be kept at a temperature of 15°C for 8-12 hours, after which the material can be examined microscopically for evidence of germination. As soon as a sample shows evidence of germination, some of the telial material can be used to inoculate the aecial host and the remainder can be used to observe stages in teliospore germination.

- Teliospore germination--Free-hand sections of telia will show teliospores in several stages of germination. The material can be stained with cotton-blue better to demonstrate stages in the development of the promycelium and basidiospores.
- Inoculation of the aecial host--Seedlings of *Yucca* spp., from two weeks to six months old, are used. The leaves are wetted, rubbed gently between the thumb and index finger, then sprayed thoroughly so that a film of water is left on the leaf surface. The plants are then

inoculated as indicated in Figure 5. In order to maintain high humidity inside the plastic bag, be sure that the soil in the pot is wet. The plants are kept at a temperature of 15°C for 24 hours, after which the plastic bags can be removed. The *Yucca* plants are then kept in the greenhouse at 22°C until there is evidence of infection. Spermogonia will usually appear in about one week and aecia will develop within 10-15 days.

- Spermogonia and aecia--If enough material is available, free-hand sections of infected leaves can be made at two-day intervals to observe stages in the development of the spermogonia and aecia. Sections of the spermogonial stage will frequently show flexuous (receptive) hyphae. Stain with cotton blue for additional contrast.

Sources of Material *P. sporoboli* occurs abundantly and consistently throughout the range of sand grass, particularly in the sandhill regions of Nebraska and Colorado and in the sand dune belt around the Great Lakes. In the Lake Michigan sand dune area between Milwaukee and Sheboygan, Wisconsin, this rust has been abundant even in years that have not been favorable for development of rusts in other areas. Any mycologist who is located near the Great Lakes sand dune area or the plains and sandhills of Nebraska, Colorado, Kansas and the Dakotas can supply it.

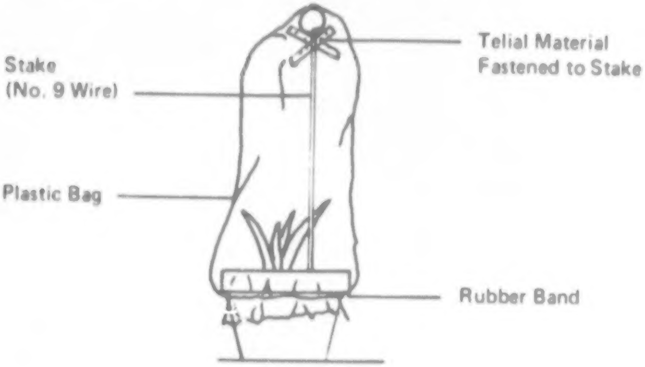

FIGURE 5 *Puccinia sporoboli* on *Yucca*.

Yucca plants are easily grown from seed.* The seeds do not require a dormant period and will germinate readily soon after maturity but will also show a high percentage of germination after a relatively long period of storage. *Yucca* plants grow slowly, so that it is possible, after one planting, to have plants with young, tender leaves over a period of six months or more. The leaves of young *Yucca* plants are crescent-shaped in cross section and thus are ideal for free-hand sectioning. Every section that is cut, when mounted in any liquid mounting material, will assume a position that presents a sectional rather than a surface view.

See also Buller, 1950; Baxter, 1962.

SMUTS

Collecting

Collections of smut fungi should be made annually or semi-annually in order to maintain an adequate supply of specimens for laboratory study. Also, teliospores are more apt to germinate when relatively fresh than if several years old, although the length of time that teliospores retain their viability varies considerably from one species to another. (See Fischer, 1951; Fischer and Holton, 1957.)

Since the smut fungi are parasites, all are to some extent coextensive with their hosts. Nevertheless, in some areas and in some years the host may escape infection, presumably because environmental conditions are not suitable. Smut fungi can be collected in arctic to tropical climates, in deserts, near sea level, or in alpine areas well over 14,000 feet high. Attempting to collect a particular species requires that the collector have firmly in mind characters of both host and fungus

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*Seed of *Yucca glauca* and *Y. elata* are obtainable from J. W. Baxter, University of Wisconsin at Milwaukee. Seed of *Y. baccata* or *Y. brevifolia* (Joshua tree) should be sought of botanists in the southwestern states, southwestern botanical gardens, or southwestern seed companies.

in association and that he seek reasonably extensive stands of the host to increase the likelihood of finding diseased individuals. Smutted plants that are naturally small may escape detection if the collector moves too rapidly through the site. All pertinent data should be recorded to facilitate making additional collections in the area in subsequent years. It is important to collect sufficient parts of the host to permit its complete identification. Frequently the underground parts (roots, bulbs, or rhizomes) are just as essential to host identification as are flowers and fruits. Also, healthy specimens should be collected to permit a side-by-side comparison with diseased plants.

Among the easiest to find are corn smut, particularly from sweet or garden corn, on which smut galls are quite obvious, and oat and barley smuts. Of the latter, smutted panicles of oat and smutted heads of barley can readily be noticed even with low incidence of infection. Cereal smuts that dwarf the host are most easily found by searching the edges of fields. Cereal smuts should be collected soon after the particular cereal crop is in head, while mature galls of corn smut may be found as soon as the first ears are ready for cooking. The abundance of corn smut increases with the increase of humidity or frequency of rain showers in any locality, whereas the presence of smuts in oat and barley depends on host varieties and on proper seed treatment. For genetic studies it may be best to obtain sub-cultures from institutions where smut studies are actively carried on. At present, the Department of Plant Pathology at Washington State University, Pullman, Wash., and Canadian Department of Agriculture Research Station, Winnipeg, Manitoba, are the two institutions where sporidial cultures of cereal smuts can be obtained.

Table 4 lists a few of the cereal and grass smuts that are widely distributed and relatively easy to collect. It includes representative species of both families whose teliospores are easy to germinate and that, as a consequence, can be conveniently studied in the laboratory. Some of these species have been studied intensively by American, Canadian, and European mycologists for many years and can be used as teaching models for studying the biology of the smut fungi in the classroom. The cereal smuts can be collected at or near harvest time in many cereal-producing states, or specimens for class use may be requested from research personnel at federal and state experiment stations. *Ustilago bullata* can be collected on a large number of *Bromus* spp. and other grasses in all of the western states and Canada. (For distribution records of these and other fungi, see Fischer, 1953.)

Storage and Preservation

The best way to collect cereal or grass smuts is to put smutted heads in an ordinary paper envelope and corn smut galls in paper cartons. If the heads or galls are green or damp, they should be dried at room temperature before shipping or storing so that specimens will not mold. The specimens may then be stored in a cool, dry place. Spores of corn smut and of the covered smuts of oat and barley will remain viable for several years, while loose smut of oat and false loose smut of barley require refrigeration to keep the spores viable more than two years.

Identification

Any serious attempt to study the biology of the smut fungi must be preceded by identification to the species level. With practice, the smut fungi can be identified to species with the help of diagnostic keys. By identifying the host to genus, the problem of "keying" a smut to species can be greatly simplified.

Teliospores should be mounted in Patterson's mounting fluid (SR-8) and their morphology and size determined by microscopic examination with oil immersion lenses. Surface markings of the various species form a variety of sculpture patterns. For example, reticulate teliospores are traversed by ridges that cross one another in a net-like fashion. Those described as echinulate are covered with very small, finely pointed spines or processes. These latter are best seen in surface or median view at high magnification and usually measure less

TABLE 4 Media and Temperatures Recommended for Certain Smuts

Smut	Teliospore Germination	Sporidial Production	Compatibility Tests
Stinking smuts of wheat (*Tilletia caries*, *Tilletia laevis* (= *T. foetida*))	18-20°C M-26, 90	15-20°C M-18-20	15-20°C M-18-20
Loose smut of oats (*Ustilago avenae*)	15-25°C M-18-20	25°C M-18-20	25°C M-90
Head smut of grasses (*Ustilago bullata*)	15-25°C M-18-20	15-25°C M-21	25°C M-90
Corn smut (*Ustilago maydis*)	20-30°C M-11, 18-20	20-30°C M-18-20	27°C M-4
Loose smuts of wheat and barley (*Ustilago nuda* (formerly *Ustilago tritici*))	19-21°C M-18-20	Although sporidia are not produced, the four cells of the promycelium can be made to separate for single haploid cell isolations by incubating the teliospores on malt agar (M-36) at 2-4°C.	Fusions between promycelial cells can be seen at the time of teliospore germination but only between those of opposite compatibility.

than 1μ. Echinulate and verrucose markings sometimes intergrade; verruculations, like echinulations, are very small but are rounded at the tips and appear as minute warts on the surface of the spores. In some of the species the teliospores are perfectly smooth. While these three are the most frequently encountered surface patterns, they are by no means the only types known in the smut fungi.

Proficiency in identifying the smut fungi may be acquired by taking previously identified specimens through the key, noting the criteria of diagnostic value that the author deemed important in constructing the key. In some species the teliospore markings are so minute that the spores appear smooth unless proper magnification, correctly adjusted lighting, and clean lenses are employed. In some finely echinulate species, teliospores from a single collection may intergrade from those that are just perceptibly sculptured to those that are perfectly smooth. Despite occasional intergradation, spore markings are an important aid to identification. Teliospore morphology, sorus morphology and ontogeny, host symptomatology, the location of sori on the host, host identity, ecology, etiology, and geographic range may all provide criteria for identification.

Teliospore Germination

Teliospores appear to be sensitive to temperature, to humidity, and to oxygen tension, different species requiring different combinations of these for germination. Technically speaking, a smut cannot even be classified with absolute certainty unless the manner in which the teliospores germinate is demonstrated, even though the teliospores of many species have never actually been so germinated.

Teliospores of many species can be germinated on a variety of media, including water. One of the commonest nutrient media employed for this purpose is potato glucose agar(M-18-20). The essential requisites for teliospore germination are water, an adequate temperature range, and a supply of oxygen. In classroom studies of any of the species listed in Table 4, none of these requisites is apt to be limiting. Temperatures required for germination of teliospores range from 5-30°C. In fact, while the temperature range for germination of some species is fairly narrow, that of others is rather wide.

For reasons unknown, mature teliospores of some species can germinate immediately, i.e., without a rest period, provided that spores are incubated at the proper temperature and that moisture and oxygen are available. Teliospores of other species may require either a prolonged resting period (up to a year) or exposure to near freezing temperatures for several months. When teliospores thus treated are placed on a suitable medium and incubated at room temperature, some germination will usually result.

The species recommended for laboratory study in Table 4 can be germinated easily at the temperatures indicated on a variety of nutrient media and generally require no rest period. For germination, teliospores should be taken (if possible) from previous unbroken sori and dusted or streaked on the agar surface. If the dishes are too thickly seeded with teliospores, or if the latter remain in clumps, details of germination are apt to be obscured. Teliospores should be examined after an overnight period of incubation to determine if germination has occurred and to observe early stages of the germinative process. This can be conveniently done by placing the dish upside down on the microscope stage without removing the lid. If the layer of agar is not too thick, the 10X objective can be lowered sufficiently to bring the germinating teliospores into sharp focus through the agar. Teliospores can also be examined by placing the dish on the stage right side up, after having first removed the lid. This technique has the advantage that teliospores can be observed under somewhat higher magnification. Its disadvantage, however, is that removal of the lid will lead almost invariably to contamination, especially in dishes that contain nutrient agar. Bacterial contamination can be suppressed by acidifying each agar plate with one or two drops of lactic acid at the time of pouring the agar.

To show the effect of temperature on germination, streak or dust teliospores of a selected species of the

Ustilaginaceae or Tilletiaceae available on potato glucose agar or other nutrient medium. Incubate the teliospores at 5°, 10°, 15°, 20°, 25°, and 30°C. Compare the percent germination at the different temperatures and note any differences in promycelial development and sporidial production. Conduct a concurrent experiment using water to germinate the teliospores. Compare the results. In placing teliospores in water, do not submerge them, as germination may be inhibited.

Sporidial Isolation

Monosporidial isolates of *U. avenae, U. kolleri, U. hordei* and similar species may be cultured *in vitro* and maintained indefinitely as pure cultures. Initially they may be obtained by using the streak method or the double-plate method or by micromanipulation.

The streak method involves streaking a sterile suspension of primary sporidia from germinating teliospores along the surface of a sterile nutrient agar plate. The single sporidial cells can then be located under the microscope and transferred by hand, or the single cells can be marked and transferred later to test tube slants when the small colonies are visible.

With the double-plate method a plate containing germinating spores is inverted over a second plate that is sterile. The ejected secondary sporidia that fall on the sterile plate are then treated as above.

Ustilago nuda does not normally form sporidia. In this case, single cell isolation is accomplished by seeding a sterile nutrient agar plate and incubating it at 2-4°C. Under these conditions, the adjacent cells of the promycelium do not form bridges, and it is possible to separate them by micromanipulation.

Isolation from Tetrads

This procedure is employed when all the meiotic products (primary sporidia) of a single teliospore are required. The method used is an extension of Hanna's dry needle procedure (Hanna, 1928). Although a simple micromanipulator can be constructed in the laboratory, it is undoubtedly more convenient to use a commercial micromanipulator when large numbers of tetrad isolations are to be carried out.

To begin with, a Petri dish is made ready to receive the germinating teliospores. This dish has a piece of filter paper at the bottom and contains three or four "van Tieghem cells" (5 mm segments cut from a soft glass tube are satisfactory). Each of the glass cells is covered with a sterile 18 mm cover slip, and the filter paper kept moist with sterile water. The teliospores are initially seeded evenly in a second dish that contains a 1 mm layer of agar. (To get a uniformly thin layer of agar, it is sometimes helpful to heat the dishes before the agar is poured.) After the teliospores have had a minute or two to settle, 5 mm^2 pieces of the agar are cut out and transferred, spores facing downwards, to the underside of the covers of the van Tieghem cells. The spores, which can now be viewed from above through the cover slip and the agar, are then left until the primary sporidia are ready for isolation.

For tetrad isolation, a microscope with a total magnification of 150X is used; the top lens of the Abbe condenser is removed to provide a wider cone of light. A chamber (Wighton, 1966) is fitted onto the mechanical stage; this chamber contains a narrow strip of filter paper that is kept moist with sterile water to prevent the sporidia from drying out during the procedures that follow. An isolating needle (Figure 6) is inserted into the micromanipulator and the tip centered in the microscopic field and then moved downwards, out of focus, well into the chamber. A cover slip with a seeded agar block is then transferred, teliospores facing downward, to the top of the chamber and above the needle. The microscope is then focussed on the lower surface of the agar and, by moving the mechanical stage, a suitable well-isolated teliospore, together with its four primary sporidia, brought into view. The needle is then raised until it touches the agar surface in the vicinity of the teliospore. Selection of a suitable teliospore can easily be tested by sliding the needle past one of the sporidia. If the sporidium detaches from the promycelium and follows the needle as it passes by, the isolation of all four sporidia should then be possible.

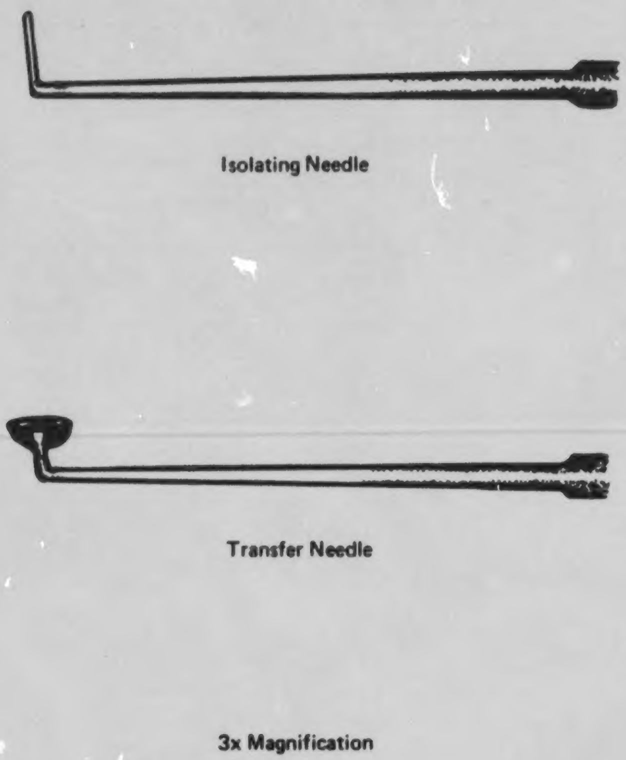

FIGURE 6 *Micromanipulator tools.*

Isolation is in the first instance achieved simply by drawing each sporidium to a position well removed from the parent spore. A suitable sketch of the optical field should be drawn to show the positions occupied by the newly-isolated sporidia and to record which of the promycelial cells gave rise to them. It is advisable not to isolate the products of more than three teliospores from a single agar block, as the dangers of drying and of contamination increase progressively with time. After this operation is completed, the cover slips are restored to their van Tieghem cells and the isolated sporidia left to form small colonies comprising ten or twenty cells.

The next step also requires van Tieghem cells, four for each successful set of isolations, prepared as described earlier. A transfer needle is fitted to the micromanipulator, centered in the optical field, and lowered into one of the two chambers. The cover slip containing the isolated sporidial colonies is placed over this chamber; a cover slip with a sterile agar block is placed over the other chamber. The transfer needle is brought up until it touches the small isolated colony, and then lowered with its adhering sporidia. The sterile agar block is then moved, via the mechanical stage, until it is above the transfer needle. The needle is then raised and the sporidia deposited on the second agar block. Contamination during this procedure can be controlled by counting the number of sporidia before and after transfer. The tip of the transfer needle can be minutely examined and, when necessary, it can be wiped by sliding it over a clean agar surface. (Do not flame the glass microtools.) Each of the four sporidial colonies of the complete set now occupies a single agar block. After a short period of growth, the colonies are visible and easily transferred to new dishes or tubes.

Compatability between Isolates

Probably the most widely-used method of determining mating reactions is the "Bauch Test." It is a convenient and reliable test for most *Ustilago* species; its usefulness depends on the formation, in compatible combinations, of aerial hyphae that, when formed in large numbers, are visible to the unaided eye. The usefulness of the Bauch test can be demonstrated as follows: a Petri dish with a 5 mm layer of nutrient agar is spotted with the six possible combinations (viz., 1 x 2, 1 x 3, 1 x 4, 2 x 3, 2 x 4, and 3 x 4) of the four sporidia that have been derived from a single teliospore. Care must be taken, of course, to exclude sporidial and other contamination and to identify the individual combinations. When the dish is examined after a day or so, either with or without a microscope, the four compatible combinations will usually have produced aerial hyphae and appear white and "fluffy," whereas the two incompatible combinations do not. In the rare case in which the Bauch test does not provide a positive result, it is possible to bring sporidia of the various combinations together by micromanipulation and, after some hours, to search directly for interconnecting bridges between sporidia of opposite mating types.

Heterothallism

Dip a flamed needle into sterile water, and while it is still wet, touch it to spores of loose smut of oats and wash them off in 10 ml tube of sterile water. Shake the spore suspension vigorously as you prepare to pour contents on a Petri dish with PSA (M-21) medium. Take cotton plug out, flame the mouth of the tube, and pour the suspension rapidly onto the agar surface. In a second or two, start tilting the dish slowly and steadily until you decant the water, place it at about a 45° angle against some object for an hour or so to drain thoroughly. You should have spores sparsely seeded at the top of the dish, becoming gradually more abundant toward the bottom. While the spores are left to germinate, fill two pots, or a flat, with soil and plant about 20 seeds of oat. Keep the pots or flat where germinated seedlings will get as much sunlight as possible, or in at least 16 hours a day of artificial light. Relatively cool nights will help to assure sturdy seedlings.

About six hours after seeding the spores, examine the dish, bottom up, under the microscope. Locate a single germinating teliospore well isolated from other spores and mark the spot. In about a day there should be a colony of sporidia visible to the naked eye. Take another tube of sterile water, flame a needle, cool it, and pick up from the marked colony a hardly visible mass of sporidia. Transfer the sporidia to the tube of sterile water, shake vigorously, and pour into a second Petri dish as before.

Finally, pick up as many sporidia as possible with the needle from several other colonies in dish #1, transfer to a third tube of sterile water, shake and pour a third Petri dish. Leave this last one flat on the table to settle for a few minutes, then decant the water and wrap the dish in plastic sheet to prevent drying. Dish #3 will produce inoculum that, when injected into oat seedlings, brings about infection.

Examine dish #2 within an hour after seeding to find a single sporidium well isolated from others, mark the spot, wrap the dish in plastic sheet, and leave until the marked colony is visible. In the meantime, place a drop of sterile water on the marked colony in dish #1, stir it gently but well with a rotary motion of the needle. Within 12-18 hours take the cover of the dish off and examine the sporidial layer of the marked colony under the microscope. You should see sporidial fusions if the mixing was properly done.

As soon as the marked sporidium in dish #2 has multiplied into a visible colony, transfer as much of it as you can (without touching any other colony) into a tube with sterile water, shake, and pour a fourth Petri dish in the same way as you did the third. This will produce inoculum that originates from a single sporidium. When injected into oat seedlings there will be no infection.

When your oat seedlings start producing the third true leaf, they are ready to be inoculated. Pour a test tube of sterile water into Petri dish #4, stir the sporidia with your (clean) finger and drain the suspension into a small beaker or cup. Draw this suspension into a hypodermic syringe, thrust the needle into the center of a seedling stem at the soil level, pointing the needle at an angle towards the base of the seedling, and force suspension in until a drop comes out from the leaf whorl. Inoculate half of the sturdier seedlings with this inoculum and the other half with inoculum from Petri dish #3. Label the inoculated seedlings appropriately and let them grow until they head out.

Mono-teliosporic Cultures

Streak or dust teliospores of *Ustilago maydis* on potato glucose agar; incubate at 20-30°C. With the 10X objective locate individual teliospores in the process of germination, and by means of a wire "biscuit cutter" isolate these to agar slants. Germinate surface-disinfected corn seeds, carefully harvesting the seedlings when they are about 1 cm long. Clip the tips of the coleoptiles to expose the tissues surrounding the plumule and growing point. Place a dozen or more seedlings in sterile test tubes and flood with an aqueous suspension of sporidia taken from the agar slants. Place the tubes under a partial vacuum for five minutes, using a water aspirator. Drain the inoculum and plant the seedlings in sterilized soil or vermiculite. Incubate for 10-11 days at approximately 85°F. This method of inoculation, devised by Rowell and DeVay (1953), should yield a high percentage of smutted plants.

Cytological Methods

Many methods have been used in staining fungal nuclei. For the nuclei of the smut fungi (which are very small) the following are among the more successful: Safranin and fast green; Iron haematoxylin; Gentian violet; and Feulgen. The feulgen stain, which is DNA-specific, is coming into ever wider use and has given good success in staining nuclei and chromosomes of *U. kolleri*.

Teliospores of *U. kolleri* are germinated for 17 hours at room temperature in Petri dishes. Selected 18 mm cover slips (i.e., with measured thickness falling between .15-.17 mm and, therefore, suitable for use with oil immersion lens) are very lightly smeared with Mayer's egg albumin, inverted over the germinating spores, and after a suitable interval (a few seconds) are withdrawn and air-dried for 10 minutes. The cover slip and its adhering spores are then rinsed for five minutes at room temperature in 70% ethyl alcohol. This is followed by five-minute rinses, also at room temperature, in 50% alcohol, in distilled water and, finally, in 1 N HCl. The adhering spores are next hydrolyzed for 12 minutes at 60°C in 1 N HCl, and then stained 15-30 minutes in the feulgen reagent (SR-11). The stained material is then rinsed four times (two minutes each) in tap water. The cover slip is then pressed firmly to a microscope slide in order to "squash" the stained material. Following this, the cover slip is immediately floated off in absolute alcohol and then replaced, using a drop of mounting medium (Gurr's neutral mounting medium, or Euparal). The preparation should be dried for 24 hours in a dust-free dark chamber before being studied under oil immersion.

18 Homobasidiomycetes

COLLECTING*

North America has one of the richest and most diverse floras of higher fungi found anywhere in the world. Some of the more common plant communities and the most prominent fleshy fungi of each are discussed in the following section.

Pine Forest or Pine-Hardwood

Pine forests have an associated fungus flora markedly different from that of most other communities. In the spring, under pines or in mixtures of aspen, white birch, pine, and balsam one often finds large numbers of such fleshy Ascomycetes as *Morchella*, *Discina*, and *Caloscypha*. After this flush of fungi has gone, there is a long period when few fleshy fungi appear above ground; in some regions, however, particularly in Idaho, species of *Rhizopogon* may be abundant during this "dead" interval.

The late summer and fall crop that features many species in abundance has three outstanding aspects.

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*Mention is made here of fungi in groups other than Basidiomycetes that may be encountered under nearly identical collection conditions.

First is the appearance of *Suillus* (Boletaceae). There are over 50 species in this genus and many are highly prized as edible fungi. During rainy summers these species may begin to appear as early as July, especially in the Rocky Mountain region. *Suillus granulatus* (which, in the Rocky Mountains, may be a summer species) favors white pine and is an exceptionally good species for demonstrating the anatomy of the glandular dots on the stipes. Because it is frequently abundant when classes are starting in the fall, *Suillus* is an excellent genus for class work on fleshy fungi. Both the constancy of diagnostic features and the variability in the species can be readily demonstrated. These mushrooms also show an association with pine in a very sharply demarcated pattern in patches of deciduous woods around pine plantations.

The second aspect of the fall crop is the distribution pattern of basidiocarps within a habitat. The common patterns for *Suillus* species and most other fleshy fungi are solitary, scattered, gregarious or cespitose. A number of "small" species in other genera, however, produce hundreds or even thousands of basidiocarps, often so close together that the forest floor is almost paved by them over a sizeable area. Species of *Mycena* are examples.

Lastly, there is the question whether the association of the fungus with the pine is direct or indirect. It should be pointed out to classes that it is necessary to synthesize mycorrhiza using a particular conifer in the laboratory if it is to be shown positively that a given species forms mycorrhizae. Many fungi are specific for the conifer-duff type of habitat, and some species may actually be parasitic on other species that form mycorrhizae with pine. Thus, a number of indirect connections are possible.

Poisonous species common in pine country are *Amanita muscaria*, *A. bisporger-verna* complex (deadly), *Lactarius rufus*, *Inocybe* spp., *Rhodophyllus* spp., and particularly some *Tricholoma* spp., which are often very abundant.

Other Conifers

Larix is the outstanding genus as far as fungal associates are concerned, especially species of *Fuscoboletinus* spp., *Suillus* spp., and *Hygrophorus speciosus*. *Pseudotsuga menziesii* (Douglas fir) is associated with *Suillus lakei*, *S. caerulescens*, *Chroogomphus tomentosus*, and *Gomphidius oregonensis*. *Picea* and *Abies* stands usually produce such a melange of fungi that a characteristic floral aspect may not seem consistent from one year to the next. These forests, as well as forests of Douglas fir, may have fungi fruiting in them throughout the year. *Tsuga* (hemlock) forests support a host of species, both terrestrial and lignicolous. The smaller fungi growing on old moss-covered hemlock logs make a fascinating project for students of ecology interested in microhabitats.

Sequoia (coast redwood) and *Thuja* habitat supports a characteristic flora in the fall consisting of *Lepiota*, *Collybia*, *Ramaria*, and *Clavaria* spp. Species of *Crinipellis* frequently abound in early summer, after a few days of rain and high humidity, on dead lower branches of *Thuja occidentalis*. In the *Thuja plicata* forests of the Pacific Northwest, species of *Lepiota* are often abundant in the fall, and *Geastrum* spp. may be numerous at times.

Birch-Aspen

This is a characteristic type so often mixed with *Abies* and *Picea* that one is tempted to treat the mixture as a distinct unit. However, pure aspen, birch-aspen, and pure birch stands are so frequent that they deserve treatment as a group. Cottonwood and balsam-poplar stands are distinct sub-types, complicated by stream border and swamp vegetation. Three major fruitings of fungi occur in the aspen associations: spring, summer, and fall.

The spring flora features species of *Helvella* and *Morchella*, *Discina perlata*, *Peziza badia*, *Tubaria* spp., *Galerina autumnalis*, *Pleurotus ostreatus*, *Clavicorona pyxidata* (which will carry over into the summer if the weather is cool), *Caloscypha fulgens*, and *Kuehneromyces vernalis*. This flora is similar to the early spring pine forest flora and many of these species are characteristic of the "snow bank" flora of the Rocky Mountains.

In the summer, *Tylopilus chromapes*, *Cantharellus cibarius*, *Lactarius vellerius*, *L. controversus*, *Amanita brunnescens*, *Inocybe lacera*, *Crepidotus mollis*, and *Leccinum oxydabile*, as well as a number of *Russula* spp., are abundant if the weather is wet.

The fall crop is the most plentiful and consists of a motley assortment, notably species of *Cortinarius* and *Amanita* and many species of *Crepidotus*. The fall crop reaches its maximum as to both species composition and quantity of basidiocarps if heavy rains come in late August or early September, an important point for mycology teachers to keep in mind. Under the above conditions, many of the more interesting fungi occur in the more barren habitats, such as sandy hillsides and stands of aspen on soil so poor that even the aspen is stunted.

Beech-Maple-Birch-Hemlock

This is the northern hardwood type, often mixed with hemlock. It supports a richer flora than the aspen-birch areas and the seasonal pattern is a spring, summer and fall sequence with the summer flora often very spotty. The spring flora features several morels, *Mycena* spp., *Tricholomopsis platyphylla*, *Xeromphalina tenuipes*, *Pleurotus ostreatus*, and, late in the spring or early summer, *Polyporus squamosus* and *P. umbellatus* (around old beech trees). After the morels, the terrestrial fungi fade out but the lignicolous species become prominent and offer the best choice for class material at this time of year.

The change from the spring to the summer flora is very abrupt; in the latitude of Ann Arbor, Michigan, for example, the changeover comes around or just after the Fourth of July. Its beginning is featured by species of the section *Hygrocybe* of *Hygrophorus*, as well as species of *Lactarius*, *Amanita*, and of *Agaricus abruptibulbus*. This phase is very dependent on hot, humid weather; if the latter does not prevail, the species may not fruit before early fall. The summer species are very sensitive to the vagaries of the weather, which is one reason mushrooms have the reputation of being so erratic. But they can also be erratic during a good season, and there is no substitute for coming to know your collecting area in great detail.

Oak-Hickory

The oak-hickory miscellaneous hardwood stands represent one of our richest habitats. Oak stands produce some of the best *Cortinarius* collecting in the fall season, if ample rains occur in late summer, and support many large and conspicuous fungi. Species of *Boletus*, *Russula*, *Lactarius*, *Inocybe*, and *Amanita* prevail in the summer in second growth woods. The dark, shoestring-like rhizomorphs of *Armillaria mellea* may be collected at any season beneath bark of stumps or fallen trunks of hardwoods, particularly oaks.

Soft-Maple Swamps

Soft-maple swamps are undergoing such an ecological change due to the ravages of the elm disease that there seems little point in discussing them in detail except to point out that, for a while at least, *Flammulina velutipes* and *Pleurotus ulmarius*, both cool-weather species, are apt to be common.

Special Regions

There are certain regions in North America that deserve special comment. First is the area generally referred to as the Pacific Northwest. Here endemism in fungi is extremely high. Among its features is the frequent occurrence of gasteromycetous fungi directly related to Agaricales. *Endoptychum*, *Thaxterogaster*, *Macowanites*, *Gastroboletus*, *Weraroa*, *Nivatogastrium (Secotium)*, *Archangeliella*, *Montagnites*, and *Brauniella* are examples. It is also the region with the largest number of species of hypogeous Basidiomycetes--*Gautieria*, *Rhizopogon*, *Martellia*, *Chamonixia*, for instance. It is characterized by a great diversity of species as well as a large number of basidiocarps per species.

The Rocky Mountains generally are noted for their "snow bank" mushroom flora, the tremendous number of species associated with *Picea engelmanii* and *Abies lasiocarpa*, and the fact that the species of *Suillus* usually fruiting in the fall in the Great Lakes area are here a major element of the summer flora (especially in *Pinus contorta* areas).

The Great Lakes region has a most interesting mushroom flora that is in the process of being documented, but critical data from such areas as northern Minnesota are almost nonexistant.

The flora of the Southern Appalachians is now fairly well known through the efforts of Coker and his students at Chapel Hill, North Carolina, and L. R. Hesler at Knoxville, Tennessee. But this flora presents an almost endless field problem because of its richness in species and the sporadic pattern of their appearance, often only a few basidiocarps at a time.

Collecting Techniques

Mushrooms and other fleshy fungi should be brought back to the laboratory in good condition for immediate study, photography, isolation, or preservation as class material. Such phenomena as chemical development of staining reactions, ephemeral morphological and anatomical characteristics, and other details can best or only be observed in the fresh condition. The technique commonly accepted by most American collectors of mushrooms has the following advantages: 1) It is adjustable to the specimens in any quantity; 2) it protects the specimens from each other to a reasonable extent; 3) in hot weather, precautions can be taken against undue sweating; 4) specimens reach the laboratory in proper condition for photography; 5) dried specimens show the characters peculiar to the species. Some collectors prefer to photograph the specimens in their natural habitat.

A large, shallow open market basket is highly recommended for collecting large fleshy fungi, particularly mushrooms. English wicker baskets or baskets made of split oak or ash are durable and satisfactory. Sheets of waxed paper (transparent, about 12 x 12 inches), newspaper, or paper bags are carried along for wrapping specimens. The specimen is carefully placed in the bag or is rolled into the paper, the ends of the paper twisted and closed, and the whole placed upright in the basket. Prepared slips of paper should be included as field labels to note substrate, type of habitat, location, and date. When collecting inky caps (*Coprinus*), the top of the paper wrapping whould be left open in order to minimize autodigestion. One or two large tin cans or quart berry boxes may be carried in the collecting basket for holding smaller specimens that may otherwise be crushed. Small cardboard boxes may be used for small puffballs. Plastic bags are undesirable for fleshy fungi, since such bags act as temporary moist chambers and favor rapid decay of soft specimens.

A necessary tool for collecting large fungi is a sheath knife with a blade about six inches long, which may be carried on the belt. It should be sturdy and sharp enough for cutting wood, or it may be used for digging specimens from soil. Other necessities are a 10X hand lens and a pocket compass for use in unfamiliar areas. One general rule for collecting is never to collect more material than can be taken care of properly upon return to the laboratory.

The collection of large woody or tough leathery fruit bodies for class use poses less of a problem, except for space, than do the fleshy forms. Less care is needed to prevent crushing. A hand axe may be useful for collecting large wood-inhabiting fungi. Although they require relatively little care, many of the woody fruit bodies may need to be dried after reaching the laboratory. Basidiocarps of *Fomes* may be broken more easily in the fresh state, to show the pore layers, than after being dried. In collecting wood-rotters, it is desirable to cut off a slab of wood showing the mycelium and type of rot and place it with the specimen.

CARE OF SPECIMENS

Upon return to the laboratory, the specimens are immediately put out to dry. Before drying, however, spore prints should be attempted for all mushrooms and other species of basidiomycetes that discharge their spores violently (Hymenomycetes). Take the specimen as it was removed from the substratum on which it grew, place it face down on white paper (or on glass) on a piece of newspaper. Fold the newspaper so that the fungus is sealed off from the dry air of the room, fold the ends of the packet under, and allow the packet to stand overnight. In case of mushrooms, the stipe is removed at the base of the gills or pores and the cap is placed gill side down on a piece of white paper. Many "white-spored" mushrooms actually have variously tinted spores, so white paper is essential in all cases.

After the attempt at taking spore prints has been made, dry the specimen. Use a number system for the specimens so that each preparation can be traced back to the original specimen. Specimens delivering negative spore prints may be young or sterile; in this event, try again with a different collection of the same species. Successful spore prints indicate a viable, actively sporulating specimen and can be useful in future work with the collection.

The most common method of preparing freshly collected fleshy fruit bodies for storage is to dry them with artificial heat. The specimens are placed on wire screens suspended above electric hot plates or a gas flame (less desirable). The entire apparatus may be surrounded by a fire-proof canvas that affords protection and acts as a chimney in maintaining air movement. For small quantities of material, an electric oven is satisfactory if it is set at low temperature and provided with aeration.

A newer drying method, using silica-gel, is preferable for small specimens. One needs plastic freezer dishes with air-tight covers and one or two gallons of activated silica-gel with blue indicator crystals added. The grain size should be about like that of wheat. Pour silica-gel into freezer dish to one-third full, place specimens in dish, fill with silica-gel, and put the cover on tightly. Within 24-36 hours the specimens are dry. When the indicator crystals have turned white, spread out the silica-gel in a flat pan and place in an oven at not more than 225^{o}F. After the moisture is driven off and the blue color reappears, the gel is ready for reuse after cooling. This technique is simple and easy, presents no fire hazard, and the specimens are nearly perfect.

Nonfleshy fungi generally may be simply air-dried.

If museum or demonstration specimens, which will not be handled by students, are desired, freeze-drying may be used. Fruit bodies dried in this manner retain their form and other characteristics but are more delicate and fragile.

So far as is known, drying is adequate for permanent preservation of any kind of fungi. Specimens preserved in this way for more than 140 years have yielded to most of the commonly used microscopic techniques and shown the sought characteristics. Dried specimens may be stored as desired for demonstration and class use. Specimens of fragile and fleshy fungi should be stored in boxes. Other specimens may be stored in folded packets and kept in shoe boxes or in drawers in laboratory benches or cupboards.

A number of insect species find dried fungi an excellent source of food and must be guarded against by placing in the box or packet a small quantity of naphthalene or paradichlorobenzene crystals. It is best not to mix these two crystalline chemicals since when mixed they tend to fuse and at least partially lose their effectiveness. A lump of material is harder on the delicate and brittle fruit bodies than are crystals. Since these substances are highly volatile, they must be replaced at intervals of not more than a year.

EXAMINATION OF SPECIMENS

Two techniques are used to show anatomical fruit body characteristics required for identification purposes: crush mounts and sections. The simpler of these is merely to tease out a bit of the hymenium. This may be mounted in a drop of 70% alcohol, which is then drained off or allowed to evaporate almost to dryness. A drop of 3-10% KOH solution is then added, followed by a drop of 0.1% aqueous phloxine, which latter increases the contrast of otherwise hyaline structures. For certain species it may be best to substitute a drop of lactophenol with cotton blue (SR-1) for the KOH-phloxine (SR-13). To another preparation of the same fungus, after air bubbles have been driven off with the 70%

alcohol solution, a drop of Melzer's reagent (SR-14) should be added. Development of a blue color will indicate amyloid walls in the spores or certain hymenial elements. If a brown or dark red-brown color is developed, the reaction is referred to as pseudoamyloid or dextrinoid. For the gilled fungi the modern standard reference, Singer (1962), uses some of these reactions critically. Singer's book and other pertinent monographs list a number of other chemical tests that should be made in certain genera. Modern treatments of other groups place much emphasis on these reactions, which should be better known.

In addition to staining techniques and chemical tests, analysis of the hyphal system is coming into favor in the study of the Polyporales. Special techniques of teasing out hyphae and determining their type and arrangement have been described in the recent literature of the pore fungi. The arrangement of the hyphae in the hymenial area can be determined by studying stained sections prepared by hand with a fine razor blade, or cut with a freezing microtome.

See also Smith, 1949; Henderson *et al.*, 1969.

HYMENOMYCETES

Thelephoraceae (Stereum)

Several species of *Stereum*, the commonest and most abundant genus of this family, serve well to represent the characteristics of the group. In most areas of the world, specimens of *S. hirsutum* are relatively common and easily recognizable. In most areas of North America, however, this species is either absent or rare, so *S. ostrea* or some other species must be substituted.

In the United States, few fungi are more widely distributed or more easily recognizable than those that belong in the complex known as *S. ostrea*. The basidiocarps grow on many different species of decaying, dying, or dead hardwood trees, and on stumps and logs. Basidiocarps appear throughout the year and usually are in satisfactory condition for morphological study even if collected during the winter. However, they are in good spore-producing condition during summer and autumn, so specimens collected during those seasons will be of greatest use. Basidiocarps are usually prepared as herbarium specimens by being dried in freely circulating air.

Basidiocarps of *S. ostrea* are easily recognizable because they rather closely resemble those of *Polyporus versicolor* but have a smooth, rather than poroid, hymenial surface. In addition, the compactly fleecy upper surface is not particularly variegated, as is that of *P. versicolor*, but is more nearly a uniform pale grayish buff, brownish, or gray. Some specimens may have a gray tomentum near the basal edge of the upper surface and a buffy tomentum toward the growing margin. The buffy, brownish, or gray color is inherent in the matted, fleecy tomentum. The upper surface of the basidiocarp, beneath the tomentum, is smooth, firm, and chestnut brown. The hymenial surface (lower surface) usually has a dull creamy buff or grayish buff color.

The basidiocarp of *S. ostrea* is definitely tough-leathery, even approaching woody, but somewhat flexible. Those that grow on the upper surface of a log may become more or less funnel-shaped, with the matted tomentum appearing on the inside surface of the funnel and the smooth spore-bearing surface on the outside. Ordinarily the funnel is not completely formed, or at least a definite fusion line shows where the two edges of the basidiocarp have met. Basidiocarps that develop along the sides of logs or stumps may fuse laterally with one another to form conspicuous shelving structures that extend laterally for many centimeters without interruption.

In section, the basidiocarp can be seen to have a lower, hymenial region of more or less vertically arranged elements, a central context of horizontal elements, a dense upper surface layer, and a tomentum of loosely-growing, more or less flexuous hyphae. Most of the hyphae of the basidiocarp appear to have rather thick walls, and many thick-walled hyphae can be traced from the context of the hymenium.

Cultures of *S. ostrea* can be obtained very readily from spore deposits from fertile basidiocarps. As basidia of *S. ostrea* do not all develop simultaneously, good spore deposits can be obtained from a particular basidiocarp at any time during the season of active growth. For best results, isolation should be attempted within two or three days after the specimen has been collected, preferably within one day. Usually the spore print will appear within a few hours after a portion of the basidiocarp is suitably affixed, hymenium facing downward, above a sterile surface such as agar. The deposit thus obtained may then be transferred in mass to a fresh sterile agar slant for use as a stock culture. Single spores may be isolated by streaking the mass of spores on the surface of a very thin layer of cornmeal agar, from which they should be transferred at the earliest possible moment after they have germinated.

Cultures of *Stereum* are readily obtainable in the field by a method described by Davidson and Hinds (1958). Required materials include large sterile agar slants, an ordinary steel table knife evenly rounded at the blade tip and ground off along the sides so that the blade will fit into the test tubes, a transfer needle, a bottle of alcohol, and a gas burner or alcohol lamp. The knife is sterilized by dipping it into the alcohol and flaming it. It is then inserted into the agar slant in the test tube in such a way that, as the knife is withdrawn, a layer of agar can be drawn along with it and plastered against the tube wall directly opposite the slanted area of the agar. After the agar is plastered against the tube wall, a small piece of basidiocarp (10 mm^2) is cut out and placed with the hymenium downward near the end of the flamed knife blade. The blade is then inserted into the test tube and the square of basidiocarp pressed firmly against the mass of agar opposite the slant. If the tube is placed in a horizontal position so that the hymenial surface of the basidiocarp is directly above the agar slant and facing it, a spore print will appear, usually within one day.

Cultures of *S. ostrea* are maintained easily on malt agar. Cornmeal agar is also satisfactory, although growth may be less profuse. Development of the cultures takes place most satisfactorily at approximately 25°C, but they should be stored in a refrigerator at approximately 12°C if they are to be kept over a long period. Cultures maintained under refrigeration may remain in satisfactory condition for a year without being transferred; for storage up to five years, they should be placed under a layer of sterile mineral oil.

On malt agar, growth of *S. ostrea* is moderately rapid; cultures incubated at room temperature are in good condition for study within one week after inoculation and remain in satisfactory condition for several weeks. According to Humphrey and Siggers (1934), optimum growth is at 28°C and growth is inhibited at 40°C. Herrick (1939b) found that *S. gausapatum* grows poorly at 5°C and 35°C, and that most profuse growth occurs at 25°C. A culture may be incubated in darkness and will grow as a white, downy mycelial mass that exhibits a moderately-developed buffy color after a few days. If the culture is exposed to light, the color will become more pronounced. Different isolates of *S. gausapatum* have been shown to vary in color and texture (Herrick, 1939a). On gallic acid agar and tannic acid agar (M-39), a strong extracellular oxidase response is indicated by the blackening of the agar beneath and surrounding the mycelial mat.

See also Burt, 1914-1926.

Polyporaceae

The general methods of preserving and using fruit bodies of the polypores in the laboratory are so well known that little need be added. It is necessary to use dried fruit bodies almost exclusively; with care the same material can be used for several years. Differences between selected genera and species are easily observed in this group and the instructor may wish to have many species on hand to use in a taxonomic study.

Common genera or species found principally or only in coniferous woods are: Certain species of *Poria*, *Lenzites saepiaria*, *Ganoderma tsugae*, *Polyporus abietinus*, *Fomes pinicola*, *F. pini*, *F. annosus* (mostly in thinned plantations). Others found principally or entirely on deciduous wood are: *Poria* spp., *Lenzites trabea*, *L. betulina*, *Daedalea confragosa*, *D. quercina* (on oak), *Polyporus versicolor*, *P. conchifer* (on elm), *P. betulinus* (on birch), *P. pargamenus*, *P. picipes*, *P. frondosus* (on ground around stumps), *P. cinnabarinus, P. gilvus, P. arcularius, Fomes applanatus, F. fomentarius, F. rimosus* (on black locust). The instructor may wish to select species for study depending on his interests and availability of material.

Polyporus conchifer is a good species to use for illustrating a unique method of asexual reproduction in Basidiomycetes and conidial dispersal by raindrops. This species seems to be the only one producing cupulate fructifications in early stages of development on dead twigs of elm in late summer or early autumn. Inside the cups conidia are produced in abundance, imbedded in a copious gelatinous matrix that hardens on drying. Later, the shelflike pore layer grows out from the side of the cup and forms the familiar conch-like basidiocarp. Dried young specimens are satisfactory if fresh material is not available, but old mature specimens, with fully developed pore-bearing brackets, are not satisfactory.

The splash cup mechanism may be demonstrated in the laboratory by allowing drops of water to fall from a height of a few feet into the vase-shaped cups containing the conidia. Some of the droplets splashed from the cup should be collected and examined for the presence of conidia.

Cultures of polypores can be obtained from spore deposits, from internal tissue of young fruit bodies, or from wood in the immediate vicinity of the fruit body. Malt extract has been used more commonly than other media but some others are satisfactory.

See also Overholts, 1953.

Boletaceae

The boletes should be studied while fresh, and the instructor must depend largely on fruit bodies collected in the forest. Many of the boletes form mycorrhizae with trees and for this reason may occur in abundance in specific types of forests following a period of rain. A specific example is *Gyrodon merulioides*, which occurs in association with ash trees and is believed to be mycorrhizal with them. The same general rules and techniques of collection and preservation of fruit bodies in the dry state for class use apply here as for other fleshy fungi.

Some species of boletes appear to be particularly susceptible to parasitic attack by other fungi. *Sepedonium chrysospermum* is common on several species and is often severe enough to cause abortion of most of the young fruit bodies in local areas. Parasitized fruit bodies may be found year after year in the same spot. *Sporodinia grandis* is also common on over-mature fruit bodies of boletes.

Color Changes Examples of color change may be demonstrated with certain boletes. The flesh of the stipe and pileus of *Leccinum aurantiacum* is normally white but turns purple black when cut. The flesh of *Boletus subvelutipes* turns pink, blue, and yellow when exposed to air and *Gyrodon merulioides* turns blue when the flesh is cut or broken. A macrochemical color change can be demonstrated with *Leccinum scabrum* by adding a drop of alcoholic preparation of gum guaiac, which turns the flesh blue. A positive reaction to gum guaiac indicates the presence of extracellular oxidase.

Culture The identity and specificity of the fungi entering into mycorrhizal union is still under investigation; in recent years cultural studies of Boletaceae are under way to help. Although many species are not strikingly different from each other in culture, there are macroscopic and microscopic characteristics that indicate presence or absence of affinity between genera and species. There are, for example, differences in the rate of growth, presence or absence of clamps in the hyphae, and position of the clamps. Macrochemical reactions characterize certain genera or species.

The production of carpophores in the laboratory by such species as *Phlebopus sulphureus*, *Phlebopus lignicola* (Pantidou, 1961, 1962), *Suillus rubinellus* (McLaughlin, 1964), *Xerocomus badius*, and *Xerocomus illudens* (Pantidou, 1964) opened new lines of research. So far, the production of carpophores in culture is not consistent and the factors necessary for normal production of fruit bodies are unknown.

When collecting for culturing, it is advisable to pick up young fruit bodies, which are therefore less liable to be associated with other fungi, bacteria, or yeasts. If possible, mature fruit bodies should be included in the collection, since often there are significant changes during the development of the carpophores, and all stages are necessary for accurate identification. Each fruit body should be wrapped in waxed paper to keep it clean and the whole collection of what is thought to be one species should be placed in the same paper bag.

Culturing should be done the same day, if possible, particularly if the fruit bodies are older. Young fruit bodies can be kept for three or four days in the refrigerator, and often fruit bodies that have been three or four days in transit will grow well when cultured. For culturing, a fine pair of tweezers and a dissecting knife are sufficient. Break the fruit body open, preferably with the fingers, trying not to touch any surface inside. The exposed surfaces of the fruit body are usually sterile. If the fruit body is a rare species, some special attempts should be made to find and culture healthy tissue by breaking the fruit body at different points in the pileus and stipe. Most workers take the tissue for culture from the point where the pileus joins the stipe, but internal tissue from any part of the fruit body grows well. The size of the cultured piece is significant. Pieces smaller than 5 mm^2 may not grow because the hyphae on the surface of the cultured piece die during cutting.

If the cultured tissue is placed flat on the agar (M-40), growth rarely occurs, whereas tissue placed so that half is immersed in the agar and half projects into the air usually grows well. Almost invariably growth starts from the part of the tissue above the agar. The first visible growth usually appears in three to five days, partly depending on the species concerned. The cultures are usually grown at room temperature, which varies between 20°-$28^{\circ}C$ for most of the year. In many species, growth is better and cultures are more uniform when grown in the dark than in the light. For the study of cultural characteristics, to be used in taxonomic or mycorrhiza work, the isolates are grown in Petri dishes in two sets, one to be kept in the dark, the other in the light.

Demonstration of clamps in the Boletaceae can be done with cultures of most species of *Boletinus*, or some species of *Suillus*. *Gyrodon merulioides* is also a suitable species, since its mycelia are frequently clamped and three clamps are often formed at the points of branching, one on the main hypha and one of each of its two branches.

Preservation of cultures of boletes is rather difficult, because most species die in a very short time. The cultures must usually be transferred to new medium every couple of months, although this is a laborious procedure. In the last few years, efforts to preserve the cultures under oil for longer periods of time seem promising. Some species thus treated proved viable after remaining under oil for over a year. However, when subculturing, the oil should be drained well from the tube, and to secure growth the whole mat should be cut in pieces and plated into a Petri dish. The cultures under oil are kept at 10°C.

See also Smith and Thiers, 1970.

Agaricaceae

For study of agarics there is no completely satisfactory substitute for fresh basidiocarps in all stages of development. Since this can seldom be attained, the instructor must either use fewer examples or resort to dried material. Fresh fruit bodies are usually available as commercially grown mushrooms that may be purchased at a grocery. The larger fruit bodies, if left in a moist chamber at room temperature for a day or two, will increase in size, mature, and produce basidiospores in abundance. (Note: *Agaricus bisporus* forms only two spores per basidium.) All stages in the development of the basidiocarp, basidia, and spores may be studied, which makes this material highly desirable for class study even though other species may be available.

Spore liberation from a fresh fruit body may be demonstrated using a strong beam of light. Any freshly gathered agaric of moderate size is suitable. Take a good-quality glass museum jar, rectangular in horizontal section, fitted with a cork or wooden lid. Having removed the stipe, pin the pileus to the lid so that it is oriented naturally. Place in a dark room and, with a suitable system of lenses, arrange a strong parallel beam of light to pass through the jar 2-3 cm below the pileus. The spores can be seen as sparkling motes because they scatter the light as they fall steadily through the beam.

The number of spores released from a fruit body may be demonstrated by placing a fresh pileus on a plate of glass under a beaker for a known time (e.g., two hours). Then suspend the spores from the spore print in a known volume of water and estimate their number by counting on a haemacytometer. The volume of water can be adjusted to give a suitable concentration for counting. From the data, calculate the rate of spore liberation per minute. Using strictly comparable sporophores, it is instructive to study the rate of spore liberation at a low temperature (e.g., $10^{o}C$) and at a high one (e.g., $20\text{-}25^{o}C$).

See also Kauffman, 1918; Krieger, 1967.

GASTEROMYCETES

Collecting puffballs and their allies requires much the same techniques as for other fleshy fungi. Fructification usually occurs during summer and autumn, but some species have a more restricted fruiting period. *Calvatia gigantea*, for example, usually occurs in southern Michigan during the first half of September.

Most puffballs occur in areas of rather sparse vegetation, in pastures, fields, or open places in woods. Characteristic of grasslands and lawns are a number of species of *Lycoperdon*, *Calvatia*, *Bovista*, *Bovistella*, and *Cyathus stercoreus* (mostly on manure). In forested areas and open places in woods, other species are common: *Lycoperdon perlatum*, *L. pyriforme* (often found in abundance on logs or stumps), *Scleroderma aurantium*, *Crucibulum vulgare*, *Geastrum* spp., and *Astraeus hygrometricus* (in sandy soil).

Secotium and *Tulostoma* are characteristic genera of open sandy regions. In the semi-arid southwestern part of the United States several unusual genera are characteristically present: *Podaxis*, *Phellorina*, *Battarraea*, *Chlamydopus*, and *Gyrophragmium*, which last shows morphological relationship with the agarics. The Phallales appear more commonly in disturbed areas, in lawns, under shrubbery, or at the edge of cultivated spaces.

The preferred methods of preservation and storage of puffballs will depend upon how the material is to be used. Dried fruit bodies are satisfactory for showing gross morphology and method of dehiscence for dissemination of spores. For this purpose mature fruit bodies should be selected. Very young fruit bodies do not show true shape or morphology when dried. The stinkhorns should be observed and smelled when fresh, or they may be frozen in bottles or in tight plastic bags until needed for class. The specimens should be removed and allowed to thaw out at the beginning of the laboratory period so that students may experience the characteristic odor. Other fresh puffballs may be frozen but must be studied immediately after thawing.

Cultures may be obtained from freshly collected young basidiocarps. To do so, split open the basidiocarp and take a small piece of tissue 1-2 mm in diameter from the middle of the puffball. Common media, such as malt extract agar, are satisfactory for growth of the mycelium. The piece of inoculum should be forced down into the agar in test tubes and at room temperature growth should be visible after about one week.

Spore Germination

The greatest difficulty in collecting basidiospores for germination experiments is to retain the spores in a relatively noncontaminated state. The following hints will help: a) Collect only puffballs that have fruited after *one* good heavy rain as opposed to those that appear after or during a prolonged wet spell; b) discard any sporophores that have wormholes in them; c) collect only very mature sporophores but, where possible, those wherein the ostiole has not yet opened or the peridium begun to crack; d) never crush sporophores in the field; keep them in a small shallow box. In the laboratory one can get a clue as to the relative cleanliness of a sporophore by streaking some spores onto a plate of nutrient agar. If fewer than three or four fungal or bacterial colonies appear in a few days, then that specific sporophore is worth saving. In the interim, dry the sporophores at room temperature (never with heat) for one or two days in a well ventilated place; those sporophores worth preserving can be placed intact in a deep freeze (about -18°C).

Special techniques are required to induce germination of spores of most puffballs. Use a fresh or frozen unopened fruit body, break open the peridium, and with sterile forceps remove a chunk of spore material about 5 mm in diameter. Place this in a small blender jar with about 100 ml of sterile water and blend for 30-60 seconds, taking care that the liquid does not get too hot. This spore suspension may be diluted as desired, but four different dilutions are suggested. Prepare malt extract agar, using 250 ml in each of four half-liter flasks, and allow to cool to 42°C (slightly warm when touched to the cheek). Dispense 10 ml of a different spore suspension into each flask of cooled, liquid medium, gently swirl to distribute the spores evenly, and quickly pour into sterile Petri dishes using 25 ml per dish. In this way the effects of spore concentration on germination may be tested. When the medium has solidified, inoculate four spots on each plate with the red yeast, *Rhodotorula mucilaginosa sanguinea*, and incubate at about 25°C. If contaminating bacteria or fungi are present, they should appear within five days. The time required for germination of puffball spores is 12-25 days. Spores of *Calvatia* spp. germinate slowly, whereas those of *Lycoperdon* are more rapid. Percentage germination is low, only 100-500 per million spores in the yeast co-cultures. Other species of *Rhodotorula* may be tested if the suggested species is not available.

Spore Discharge

A simple, effective demonstration of spore expulsion in *Lycoperdon* spp. can be done in the laboratory. Use mature fruit bodies that have a small, well-formed apical pore. Spores may be expelled by tapping the peridium, but a more natural method is illustrated by falling drops of water. Hold the fruit body at a slight angle several inches beneath a water faucet, or other source of slowly dripping water, so that a drop every few seconds will fall on the peridium (not the ostiole). As the dry peridium becomes moist, it becomes more pliable, and spores are puffed out by each falling drop. This method of spore discharge should be compared with that in *Calvatia, Scleroderma,* or other genera in which the peridium opens irregularly. (See, in this connection, Figure 19.)

The earth stars have a pliable inner peridium and spore dispersal that is similar to that of *Lycoperdon. Astreaus hygrometricus* is unique in having a hygroscopic outer peridium the segments of which fold over the inner peridium when dry. After becoming wet, the segments curve outward and backward and the rain drops then fall on the unprotected inner peridium. This method ensures dispersal of spores when the soil is wet. The hygroscopic nature of this fungus may be demonstrated by placing dry fruit bodies in moist chambers on wet paper towel or absorbent cotton. After class the fruit bodies may be dried and stored for reuse. If material is plentiful, the students may wish to cut thin cross sections of the outer peridium and observe differences in the tissue layers that cause them to bend when moistened.

Nidulariaceae

The fruit bodies of the bird's-nest fungi are unique in morphology and structure. The structure of the peridioles and the funiculus may be studied best in fresh fruit bodies of *Cyathus.* If dried specimens are to be used for dissection, they should be held for at least two hours in a moist chamber. *Cyathus striatus* has the most highly developed funiculus, with those of *C. olla* and *C. stercoreus* being simpler. *C. stercoreus* has the advantage of fruiting readily in culture, thus ensuring a supply of fresh fruit bodies at the desired time. *C. olla* fruits reasonably well in culture, but other species do so poorly. The funiculus of *Crucibulum* is somewhat different from that of *Cyathus.*

When dissecting the fruit bodies, cut pieces of the peridium with fine sharp scissors so that each piece bears one or more attached intact peridioles. Complete the dissection under water in a Petri dish with the aid of dissecting microscope. Hold the piece of peridium with one pair of forceps and pull gently on the peridiole with another pair, watching for the stretching of the "middle piece" and finally the explosive emergence of the "hepteron." Grasp the hepteron with forceps and pull out the funicular cord, which may be stretched along a glass slide and observed under a compound microscope.

Fresh specimens are highly desirable for demonstration of splash ejection, for peridioles of old specimens may fail to become separated from one another. Fruit bodies must be attached firmly to some base so that they will not be knocked over by water drops. This can be done by inserting three pins at an angle through the base into a large flat cork. Drops of water must fall at least 10 feet to have enough momentum to eject peridioles. Water can be delivered from a pipette held by clamps and allowed to drip slowly. Peridioles may be hurled 4-10 feet and it may, therefore, be necessary to search for them.

Not all isolates of *Cyathus stercoreus* fruit equally well in culture, and fruiting capacity should be determined before material is needed for class. Inoculate several plates of Brodie's special fruiting medium (M-53) with diploid mycelium, wrap in clear cellophane, and incubate near 25°C in darkness for 10-14 days. Then place cultures about two feet below two 40-watt cool-white fluorescent lamps. Fruit bodies should be well developed within 30-45 days, depending on the isolate.

Sphaerobolus stellatus is easily maintained in culture without any loss of fruiting capacity, provided that each new culture is started from a discharged peridiole. The use of mycelium inoculum soon leads to reduced fruiting and finally to sterility. It fruits on a number of common media but oat agar is the best of those tried. Since cultures take several weeks before being ready for class, they should be on a relatively deep layer of agar, and precautions should be taken to prevent undue desiccation.

Fruiting is dependent upon exposure to light. A dependable procedure is to inoculate each plate with a peridiole and leave in darkness for three weeks. Then place the cultures under continuous light of 50-100 ft-c from daylight fluorescent tubes. Optimum temperature is near 20°C and should not rise above 25°C. Under favorable conditions, fruiting will be continuous and peridiole discharge goes on for months, but with innate periodicity of about 12 days.

See also Coker and Couch, 1928.

CULTURE OF TRUE BASIDIOMYCETES

Cultures of most true Basidiomycetes may be obtained, by aseptic techniques, from internal tissue of young fruit bodies or from basidiospores. To obtain discharged basidiospores, a small piece of the sporulating tissue may be attached by a pin to a cork that is fastened by an adhesive to the lid of a moist chamber. Spores may be caught on sterile glass slides or on agar plates. If the specimen is small enough, it may be fastened to the lid of a Petri dish and the spores allowed to fall directly on the prepared agar plate. By rotating the lid every few seconds, or minutes as necessary, the spores may be well separated so that individual germinated spores may be located and removed by means of a special isolation needle.

If one prefers, a spore suspension in distilled water may be diluted and used to flood agar plates. One should examine the suspension under the microscope and adjust the dilution so as to give a relatively small number of spores per plate. Germination in most species occurs within two days. Single spores should be picked out while the germ tubes are still short enough to be traced to the individual spores. A small bit of agar containing a germinating spore may be cut out by means of a slender needle inserted in a suitable holder. Some workers prefer to lower a small circular cutter formed from the eye of a specially prepared fine sewing needle over the germinated spore and lift out the spore with a bit of agar. This method is simple, it can be modified as desired by the instructor, and it can be used for any spores that can be distinguished under the magnifications used. The single spores should be placed in test tubes of suitable agar for growth of mycelium. Frequently, single-spore cultures develop more slowly than do those from many spores or those started from mycelium. Multispore cultures should also be obtained at the same time and compared with the single-spore cultures.

Wood-inhabiting Hymenomycetes

When collecting wood decay fungi for culture work, it is useful to remove a large piece of wood, or a section of the branch, with a small saw and place it in a plastic bag. This material may be kept in the refrigerator for two or three days. If it must be kept longer, then the whole section of wood may be placed in the deep freeze and mycelium isolated from the wood several months later.

In isolating wood decay fungi, take small pieces of decayed wood with sterile forceps or a cork borer from the wood just beneath the basidiocarps. Malt agar (M-35) with 0.5% malic acid added does not inhibit the fungus but does check bacterial contamination. The general area of the basidiocarp as well as the basidiocarp itself should be lightly flamed before removing a wood fragment.

There are several ways in which cultures of wood-inhabiting basidiomycetes may be used in the laboratory. They may be used to illustrate various characteristics of the mycelium, or as unnamed cultures that may be identified in a laboratory exercise using published keys of Nobles (1948, 1965); in so doing, it is important to grow the cultures at 20°C. Selected species may be used to demonstrate heterothallism and types of compatibility, such physiological phenomena as responses to light or bioluminescence, and genetic studies.

In order to observe the characters of the mycelium on which identification is based, cultures of several species should be available. A suggested basic list is given below:

Polyporaceae	Agaricaceae
Coriolellus serialis	*Armillaria mellea*
Fomes annosus	*Panus stypticus*
Fomes cajanderi	*Schizophyllum commune*

Polyporaceae (continued)

Fomes igniarius
Fomes pini
Fomes pinicola
Fomes roseus
Ganoderma applanatum
Lenzites saepiaria
Polyporus adustus
Polyporus balsameus
Polyporus tulipiferae
Polyporus versicolor
Pycnoporus cinnabarinus

Thelephoraceae

Coniophora puteana
Corticium vellereum
Trechispora brinkmanni

Other species may be substituted and certain ones omitted if convenient and desirable. It is recommended that the Nobles' key be used and that malt extract agar (M-35) be used. Gallic or tannic acid agar (M-39) is needed to demonstrate presence of extracellular exidase.

One can substitute gum guaiac in place of gallic or tannic acid agar media to demonstrate extracellular oxidase. A drop of this chemical (in alcoholic solution) placed on the colony will turn blue if oxidase is present. This reaction, although not as consistent as gallic or tannic acid agar, can save the extra time involved in growing the fungus on two different media.

Species that fruit readily in culture are ideal for demonstrating the various types of interfertility, homothallism, bipolar and tetrapolar heterothallism. Other selected species may be used if a source of single basidiospore cultures is available and if the species produces clamp connections. Homothallism may be demonstrated by *Trechispora brinkmanni*. Single basidiospore cultures produce hyphae with clamp connections at the septa and have the ability to form fertile fruiting areas on agar.

Dikaryotic cultures of *Coriolellus serialis*, *Fomes cajanderi*, *Fomes roseus*, and *Polyporus adustus* form clamp connections and fruit on malt agar, although, as is true for many fungi, certain strains fruit far more readily than others. These species illustrate bipolar heterothallism. Single-spore cultures consist of simple-septate hyphae. To determine the type of interfertility, several (about a dozen) single-spore isolates from the same fruit body are grown together in pairs (about 2 cm apart) on agar plates or in tubes in all possible combinations. A few days after the cultures have grown together, small bits of mycelium are removed from the line of meeting and examined for hyphae with clamp connections. Results may be recorded on a chart with a plus sign representing presence (fertile) and a minus sign the absence of clamp connections (infertile) in each pairing. When these results are rearranged in the chart, it will be found that there are only two compatibility groups, which can be arbitrarily designated A_1 and A_2. All pairings within the same group are incompatible and all pairings between groups are compatible, i.e., they produced clamp connections. In this case, compatibility is governed by a set of alleles at a single locus. Bipolar heterothallism is also illustrated by *Pleurotus ostreatus*, *Fomes annosus*, *Polyporus betulinus*, *Auricularia auricula-judae*, *Exidia* spp., *Lenzites trabea*, and others. A sample of data for *Coriolellus serialis* is shown in the chart below, based on 16 single-spore cultures.

		A_1										A_2					
		1	2	3	6	7	8	10	14	15	16	4	5	9	11	12	13
A_1	1		-	-	-	-	-	-	-	-	-	+	+	+	+	+	+
	2	-		-	-	-	-	-	-	-	-	+	+	+	+	+	+
	3	-	-		-	-	-	-	-	-	-	+	+	+	+	+	+
	6	-	-	-		-	-	-	-	-	-	+	+	+	+	+	+
	7	-	-	-	-		-	-	-	-	-	+	+	+	+	+	+
	8	-	-	-	-	-		-	-	-	-	+	+	+	+	+	+
	10	-	-	-	-	-	-		-	-	-	+	+	+	+	+	+
	14	-	-	-	-	-	-	-		-	-	+	+	+	+	+	+
	15	-	-	-	-	-	-	-	-		-	+	+	+	+	+	+
	16	-	-	-	-	-	-	-	-	-		+	+	+	+	+	+
A_2	4	+	+	+	+	+	+	+	+	+	+		-	-	-	-	-
	5	+	+	+	+	+	+	+	+	+	+	-		-	-	-	-
	9	+	+	+	+	+	+	+	+	+	+	-	-		-	-	-
	11	+	+	+	+	+	+	+	+	+	+	-	-	-		-	-
	12	+	+	+	+	+	+	+	+	+	+	-	-	-	-		-
	13	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	

Tetrapolar heterothallism may be demonstrated in the same manner by using *Pycnoporus cinnabarinus*, *Schizophyllum commune*, or *Corticium vellereum*. Single-basidiospore cultures from the same fruit body fall into four groups, based upon their pairing reactions. The pairing reaction is determined at two loci with a series of alleles at each locus that segregate independently. Sample data for *Pycnoporus cinnabarinus* are shown in the chart below, based on 16 single-spore cultures.

		A_1B_1				A_2B_2				A_1B_2					A_2B_1		
		1	3	7	9	2	5	11	12	4	6	8	13	16	10	14	15
A_1B_1	1		-	-	-	+	+	+	+	-	-	-	-	-	-	-	-
	3	-		-	-	+	+	+	+	-	-	-	-	-	-	-	-
	7	-	-		-	+	+	+	+	-	-	-	-	-	-	-	-
	9	-	-	-		+	+	+	+	-	-	-	-	-	-	-	-
A_2B_2	2	+	+	+	+		-	-	-	-	-	-	-	-	-	-	-
	5	+	+	+	+	-		-	-	-	-	-	-	-	-	-	-
	11	+	+	+	+	-	-		-	-	-	-	-	-	-	-	-
	12	+	+	+	+	-	-	-		-	-	-	-	-	-	-	-
A_1B_2	4	-	-	-	-	-	-	-	-		-	-	-	-	+	+	+
	6	-	-	-	-	-	-	-	-	-		-	-	-	+	+	+
	8	-	-	-	-	-	-	-	-	-	-		-	-	+	+	+
	13	-	-	-	-	-	-	-	-	-	-	-		-	+	+	+
	16	-	-	-	-	-	-	-	-	-	-	-	-		+	+	+
A_2B_1	10	-	-	-	-	-	-	-	-	+	+	+	+	+		-	-
	14	-	-	-	-	-	-	-	-	+	+	+	+	+	-		-
	15	-	-	-	-	-	-	-	-	+	+	+	+	+	-	-	

In addition, complete interfertility between single-spore cultures from different fruit bodies may be demonstrated by using fruit bodies from different sources. If, for example, four single-spore cultures from one fruit body are paired with four from another fruit body and the mycelium of all pairings produces clamp connections, then it can be assumed that the two are conspecific.

See also Davidson *et al.*, 1938; Cartwright and Findlay, 1958.

Agaricus bisporus

"Home-Gro Mushroom Garden" kit, obtainable from Mushroom Supply Co., Kennett Square, Pa. 19374, at a modest cost contains everything needed to grow *Agaricus bisporus.* The mushrooms may be grown at room temperature in the light or in the dark. Carbon dioxide inhibits development. About one month is required for the initiation of primordia; after the first crop matures, new crops will develop weekly for about one month.

The regions of expansion in the carpophore can be demonstrated by placing carmine spots equidistantly along the stipe and cap at an early stage (0.5 cm high) in development. Measurement of the distances between the spots is made at various stages in development and, when graphed, will indicate that stipe elongation occurs primarily in the zone immediately below the cap, while cap expansion is predominantly marginal. To demonstrate that increase in size of the carpophore involves the uptake of dry matter, as well as water, from the vegetative mycelium, wet and dry weights at different stages in development are plotted graphically, resulting in a more or less linear curve.

The importance of the gills for stipe elongation may be demonstrated if parallel cuts are made such that the pileus extends from only two sides of the carpophore and the fruit body appears T-shaped. If the gills are removed from one side of a young fruit body (25-30 mm tall), the stipe will bend away from the side bearing the remaining gills. Such curvatures may also be obtained if most or all of the pileus tissue is removed but the gills left attached to one side of the stipe, or if the gills remain attached to one side by the veil only. Curvature of the stipe can be induced with water, ether, acetone, or ethanol extracts of the gills or by certain amino acids when they are applied unilaterally to a gill-free carpophore.

Coprinus lagopus

Coprinus lagopus occurs on horse dung and has been isolated from spores. To prevent drying of the pileus while spores are being shed, a piece of the mature cap is attached to the inside of a Petri dish lid with vaseline or tape and the spores allowed to fall on 0.3% yeast extract agar (M-63a). *C. lagopus* is a tetrapolar heterothallic species. Single-spore cultures may be isolated and a dikaryon established by mating compatible pairs, or a fruiting culture may be obtained by isolating a group of spores. In the latter case the dikaryon arises naturally.

Fruiting bodies can be obtained on a defined medium (M-71) in cotton-plugged 10-oz medicine bottles lying on their broad inner face and containing 20 ml of medium. Standard Petri dishes containing 20 ml of medium have also been used. Incubation should be at 25°C. Light is not required for the initiation of fruiting bodies, but it is necessary for normal stipe and cap formation. In the light, primordia appear between the eighth and the eleventh day, and fruit bodies mature in about two weeks. Successive crops of fruiting bodies may be obtained.

In *C. lagopus* the primordia are distributed unevenly in the Petri dish; development of some favored primordia seems to initiate a flow of nutritive materials in their direction and reduces fruiting in other parts of the culture. This flow of nutrients would explain why some attain maturity and others do not. If two halves of a Petri dish culture are separated at the time of primordium initiation, this flow of materials can be prevented and an even distribution of carpophores in the cultures will result. For this experiment, two sets of five Petri dishes are inoculated; two similar Petri dish cultures are started the previous day to indicate when fruiting is imminent in the main cultures. After about a week and a half, primordia will appear in the indicator cultures. One set of the experimental cultures is then bisected with sterile scalpel and half of each culture transferred to a sterile Petri dish. Petri dishes are incubated for about a week until carpophores mature but not so long that they deliquesce. Total dry weight of fruiting bodies is taken for each half Petri dish because cultures often differ in number and size of sporophores, yet agree in terms of total dry weight of sporophore tissue produced. In the nonbisected set of cultures, the more heavily fruited half of the culture is separated from the less heavily fruited. The data usually indicate a nearly equal distribution of dry weights of sporophores in the bisected cultures and a very unequal distribution in the nonbisected cultures.

Flammulina velutipes

Flammulina velutipes (Collybia velutipes) fruits in small clusters on decaying logs and stumps and on the bark of living trees, where it causes a white rot of sapwood. It appears chiefly in late autumn but also in other seasons. It may be isolated by plating spores on malt agar. *F. velutipes* exhibits tetrapolar sexuality.

Fruiting may be obtained on malt agar (M-36), especially if cultures are pretreated as follows: After the flasks are inoculated, incubate them for one week at 25°C in the dark and then move them to an incubator at 15°C in the light. Mature carpophores are produced four to five weeks after the start of the experiment.

Fruiting bodies may be obtained at 5°C to 20°C but, depending on the strain, results tend to be more erratic at 20°C. Light is necessary for pileus expansion but not for cap and stipe initiation or for stipe elongation. Only minimal amounts of light are necessary; 70 ft-c is more than adequate. Sporophore formation is inhibited when the cultures are maintained in sealed containers, because of an accumulation of CO_2, or when evaporation rates are too high.

Polyporus brumalis

Polyporus brumalis occurs on dead wood, usually of deciduous trees. It may be found throughtout the year. This fungus can probably be isolated from spores or tissue on malt agar. Fruiting can be induced on liquid 4% malt extract medium adjusted to pH 4.2-4.3. Temperature affects the rate of fruiting over a range greater than 10°C to 27°C; about 20°C has been used in general. The production of mature sporophores requires low humidity and light; aeration is not necessary. The necessity that humidity be kept low makes it necessary to replace water lost from the cultures. Low light intensity (40 to 75 ft-c) and high humidity induce formation of epileate carpophores.

Schizophyllum commune

Schizophyllum commune is found in nature mostly on wood of deciduous trees, rarely on coniferous wood, and occasionally on roots and stems of herbaceous plants. It is saprophytic or weakly parasitic and cosmopolitan in distribution. Isolation can be made from spores by suspending the carpophore over an agar plate for a few minutes. Spread the spores with a bent glass rod and incubate the plate at room temperature for 16-24 hours to permit germination. Single spores are isolated on small agar blocks. Malt agar may be used for isolation and maintenance of cultures. *S. commune* exhibits tetrapolar sexuality; single-spore isolates must be mated so that a dikaryon capable of fruiting results. A dikaryon may be obtained by mating single-spore cultures in pairs. Alternatively, a group of spores may be transferred from the original isolation plate and the dikaryon allowed to arise naturally.

Fruiting may be achieved on certain media (M-80). Petri dishes (90 mm in diameter) with about 30 ml of medium per plate are used; crystallizing dishes or flasks (100 ml) may also be used. Plates are incubated at 22 to 23°C, without humidity control. Light is necessary for fruiting but the intensity and duration are not critical; there is even one published report of a strain that does not require light. There is tremendous variation in the fruiting competence and time of fruiting of different strains. Carpophores may be expected at any time after the fifth day.

Two growth forms may be demonstrated by incubating some plates or crystallizing dishes standing on edge and placing others in an inverted position. The fruit body normally develops as a small globose primordium with an apical cavity, which enlarges to form a cup in which the hymenium develops. The gills arise mainly as marginal ingrowths of the cup. In those plates incubated standing on edge, the margin of the cup expands unilaterally. The gills show a positive geotrophic response and the stipe becomes eccentric. In the inverted plates, the margin of the cup expands uniformly and the stipe is attached near the center of the pileus.

PART III

ECOLOGICAL GROUPS

19 Biological Associations

INSECTS AND FUNGI

Ambrosia Fungi

The term ambrosia is used here to designate those fungi that are symbiotically associated with beetles throughout their life-cycle and serve as the major source of food for larvae and adults. The characteristic ambrosial mass is variously colored, and during the optimal period of insect activity it usually consists of a palisade of catenulate hyphal bodies that may or may not bear monilioid chains of conidia. Sometimes this palisade consists of distinct conidiophores bearing conidia terminally or laterally.

Ambrosia fungi (Figure 7) are usually species-specific in relation to their insect symboint. Occasionally one finds closely related beetles associated with the same species of fungus or a variant thereof. Ambrosia fungi have thus far been isolated only from the beetles or from their tunnels in wood. Most of these fungi are comparatively slow growing and pleomorphic. Some are able to grow at temperatures above 37°C and many have fruitlike odors.

Most ambrosia fungi belong either to the subclass Hemiascomycetes or are Fungi Imperfecti; no Phycomycetes or Basidiomycetes have as yet been isolated as ambrosia fungi. Some of the more common species are: *Ascoidea hylecoeti* associated with the ambrosia beetle *Hylecoetus dermestoides* infesting *Pinus* sp. in Germany; *Monilia brunnea* with *Monarthrum fasciatum* infesting various *Quercus* spp. in the United States; *Monilia ferruginea* with *Trypodendron domesticum* infesting *Fagus* sp. in Sweden and Germany; and *Endomycopsis fasciculata* with *Gnathortrichus materiarius* infesting *Pinus* spp. in the United States.

Ambrosia beetles belong to the families Scolytidae, Platypodidae, and Lymexylonidae. The Scolytidae, widely distributed in temperate and tropical regions, include the important ambrosia-feeding genera *Xyleborus*, *Trypodendeon*, *Gnathotrichus*, and *Anisandrus*, but many genera are not fungus feeders. The Platypodidae are mostly tropical, all are fungus eaters, and among the commoner ones are the genera *Platypus*, *Crossotarsus*, and *Diapus*.

Ambrosia beetles attack hardwoods and softwoods, the former being infested more frequently. Perhaps the most important substrate for them is timber or standing trees weakened by flooding, drought, disease, or fire. They rarely attack healthy trees or dry timber. Many ambrosia beetles infest a wide variety of trees and shrubs, but some are oligophagous. The beetles do not ordinarily attack wood with moisture content of less than 50%.

The tunnels of ambrosia beetles can be easily distinguished from those of other wood borers. A clean cut through a tunnel of an ambrosia feeder usually shows a brownish or blackish discoloration in wood around the hole, which is devoid of wood dust or frass. Entrance to the tunnel system of the beetles is a circular hole only slightly larger than the diameter of the insect's body. When beetles are active inside wood, there is usually fresh, fine wood powder outside the entrance. This powder accumulates in bark crevices, at the base of a tree or a log, or on the surface of infested material if it is lying horizontally. Occasionally this powder, along with dark brownish minute pellets of larval frass, is extruded in the form of compact cylinders (0.25 to 3.0 cm long) tightly fitting in the entrance. In all cases, beetles feed on the ambrosia fungus that covers almost the entire tunnel system.

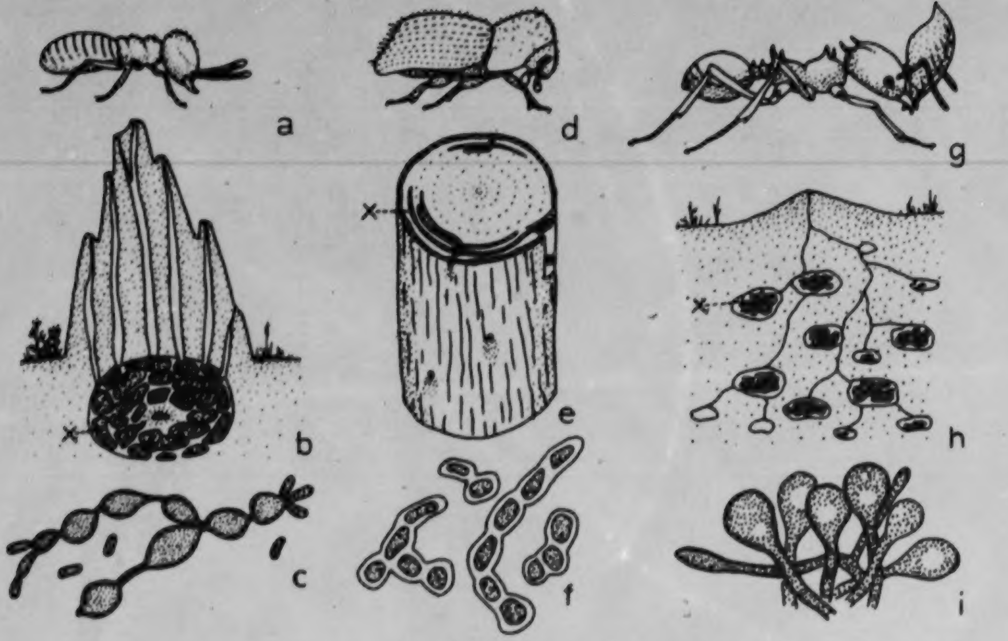

FIGURE 7 a-c, *Fungus-growing termites:* a, *worker;* b, *termitarium with fungus garden* (x); c, *cells from fungus garden.* d-f, *Ambrosia beetles:* d, *beetle;* e, *log, showing fungus tunnels* (x), *larval cells, and sawdust;* f, *ambrosia fungus.* g-i, *Attine ants:* g, *worker with piece of leaf;* h, *nest with fungus gardens* (x); i, *bromatia.*

Maintenance in the Laboratory Logs infested with active ambrosia beetles *Monarthrum*, *Xyleborus*, *Gnathotrichus*, and *Trypodendron* can be easily procured from logging areas in temperate North America from early spring through summer. A few beetles may be found in the fall. Infested logs of suitable dimensions, usually 40-50 cm long and 15-20 cm in diameter, should be washed by scrubbing under running water to remove loose dirt, mites, and eggs of undesirable insects. They should then be wiped with cheesecloth dipped in ethyl alcohol and any excess flamed away. Repeat disinfestation three to five times. Smear the cut ends of logs with petroleum jelly or, preferably, with paraffin wax to prevent dehydration and infestation by undesirable saprobes.

Although a terrarium for maintaining cultures of beetles and fungi in a relative humidity of 80-90% has been used, logs can be easily stored for one or two months in cardboard boxes lined with a sheet of polyethylene. A portion of a log containing tunnels can be sawed off, subsequently sliced aseptically with a hatchet or a chisel, and brood tunnels thereby exposed. In order to ascertain that one is dealing with true ambrosia fungi and not with secondary contaminants, the following procedure should be adopted: (1) Observe whether larvae are feeding on ambrosia in tunnels or cells; (2) determine presence of ambrosial cells microscopically before plating; and (3) ambrosia cells, or conidia, from tunnels and from mycangia where possible, should be streaked on 2% malt extract agar or potato glucose agar in Petri dishes; they should be located under low power of a microscope, periodically examined, and upon germination transferred to obtain pure cultures.

Isolation of Fungi If adults abandon tunnels before their progeny emerge, nonspecific foreign fungi soon overgrow the ambrosial palisade and kill immature beetles. It is imperative that individuals from vigorously flourishing colonies be used. For isolation of ambrosia fungi, allow beetles to crawl on sterile moist filter paper in a Petri dish for two hours. Transfer to another moist Petri dish for 12 hours; then incubate in a dry Petri dish for the same length of time. This alternate incubation in moist and dry chambers should be repeated thrice to reduce contamination. The beetles are then placed on a flamed microscope slide, their elytra removed and plated. Crush the head, pronotum, mesonotum, metanotum, and abdomen separately in a drop of water and streak the fungi, if present, on agar media. Aggregations of insect fat cells or fat globules may be confused with chains of ambrosia cells. The latter, however, are less refractive and have a cell wall.

Fungi Cultured by Ants

Ants of the genera *Atta* and *Acromyrmex* (Figure 7) are the famous Latin American leaf-cutters that are considered major agricultural pests in many areas. They occur in the southwestern United States from Louisiana to California. In addition, there is a less conspicuous fungus-grower, *Trachymyrmex*, common in the pine-oak woods of the Atlantic and Gulf Coasts and inland in places as far as southern Illinois. It formerly occurred north to Long Island, New York, and is common in the New Jersey pine barrens.

The fungus itself has not been recognized outside the ant nests, and ant nests must be found in order to secure a culture. Fortunately, the nests are generally recognizable from the low volcanic crater type of structure surrounding the entrance. Securing the material, therefore, has an ecological aspect, and this ant-fungus relationship is one of the best examples of symbiosis.

Recognizing the Ant Nest The southwestern *Atta texana* nest, when mature, has multiple craters that together form a low mound. The individual crater may vary from 10-50 cm in diameter and be up to 10 cm in height. The nests are in sunny sites, often in the neighborhood of creeks but not in places that may be flooded. The nest of *Acromyrmex (Moellerius) versicolor*, found in more semi-arid and desert areas, tends to have fewer craters that are scattered rather than forming a low mound. In both cases it is less laborious to excavate a young rather than a mature nest because of the depth and extent of the latter, which, in *Atta*, may consist of scores of chambers to a depth of 2-3 meters. Young nests are those that have only one or at most a few craters. The youngest type of nest may have one or two chambers at a depth of 10-50 cm. In all cases, the use of a shovel rather than a trowel may be anticipated. If the nest is in rocky or otherwise difficult sites, an easier site should be sought.

In hot, dry weather the ants may be inactive until late afternoon, and one should usually look for a nest to which the ants are actively bringing in cut leaf sections. The tunnel (or tunnels) leading to the underground chambers does not necessarily proceed vertically down but may meander to one side. It may, therefore, be necessary to sink a number of shafts before pay dirt is reached.

The chambers or cells with fungus gardens are like nothing else commonly found, They may be spheroidal or elliptical in section, and if the fungus garden is not suspended from rootlets, it rests on the bottom. The sides of the cell do not touch the garden and commonly there is a free space at the top. The garden looks somewhat spongy and is obviously a mold growth on discrete particles of plant leaves or stems. Under a hand lens the filaments of the fungus may be distinguished.

Collecting Once the nest has been exposed, the garden should be treated with the greatest care. With a trowel transfer as entire a garden as possible to a glass or plastic jar, avoiding as much physical break-up as possible. The atmosphere above ground will probably be drier than the garden chamber, so that wet toweling or wet sand should be introduced with the garden. In the meanwhile, ants will be swarming about and attempting to bite. They have no functional sting, and the bites in the U.S. ants, except for the soldiers in *Atta*, are of no particular consequence and do not break the skin. Keep as many ants as possible with the garden. The ants in nature are immaculate and will not contaminate. Keep the containers as near the sub-surface soil temperature as possible; in any case 25°C will be satisfactory.

Transportation and Storage The material does not keep well during transportation, particularly if it is shaken, and the sooner it can be placed in a stable place in the laboratory the better. If transporting the material by car, keep in mind that a car seat vibrates more than the floor and that the rear floor gets more shock from the road than the front floor of the car. Avoid extremely high temperatures, say above 33°C.

When the gardens are placed in a cooler place, there will probably be condensation of moisture. This will do no harm unless the garden is actually wetted. An incubator at 20°-25°C is a satisfactory place for storage. At this lower temperature the ants will not be so active.

Culturing Best results in culturing will be obtained if it can be done within the first day or so. The day of collection is satisfactory if the garden can be broken up under the dissecting microscope and inner, uncontaminated material used. For inoculum use only the clusters of inflated hyphae. These white, glistening masses of hyphae (staphylae) contrast with the spidery appearance of the ordinary hyphae. Transfer them in a quiet room, using a sterile needle. If picked up gently from a normal, inside wall of a cell of the garden, they will in most cases be uncontaminated. Transfer quickly to nearby sterile potato glucose agar or Saboraud's glucose agar plates or test tubes. Try a dozen transfers at a time in case some become contaminated. Later they can be cultured on oats or wheat in flasks (Figures 8-9).

The inflations of the hyphae and their aggregates are most characteristic of the fungi. These can be returned to the ants to determine acceptability, for they do not accept other species of fungi.

See also Weber, 1966, 1969, 1972.

Fungus-bearing Insect Galls

Some gall midges (Diptera: Itonidinae) are regularly associated with certain fungi in their plant galls. The fungi appear during early stages of gall development and usually form a relatively thick palisade of fungal hyphae lining the inner surface of the gall. They are parasitic on the gall tissue. A single fungus species usually predominates inside a gall. A particular fungus

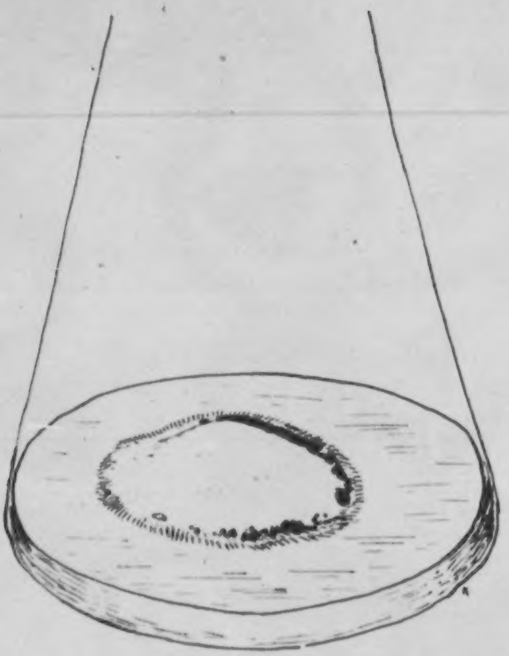

FIGURE 8 *Culture of fungus (on oats or wheat) from ant nest in flask (large white mass is hyphal mass; small circles represent white hyphal aggregations).*

may be found consistently in association with a particular insect, but the fungus may also be isolated from other substrata. In some cases, e.g., floral bud gall of *Asphondylia cytisii* on *Cytisus* spp., the fungus may be sharply delimited from the rest of the gall tissue by hyphae penetrating into the first, or at the most second, innermost layer of cells of the gall. In other cases, e.g., blister galls on leaves of *Solidago* spp., caused by *Asphondylia carbonifera*, the fungus penetrates throughout the gall tissue and it appears as if the fungus is the sole cause of gall formation.

Many of the fungus galls are caused by sap-sucking insects like *Asphondylia* and *Lasiopteva*. Experiments with insects and fungi recovered from galls of *Cytisus*,

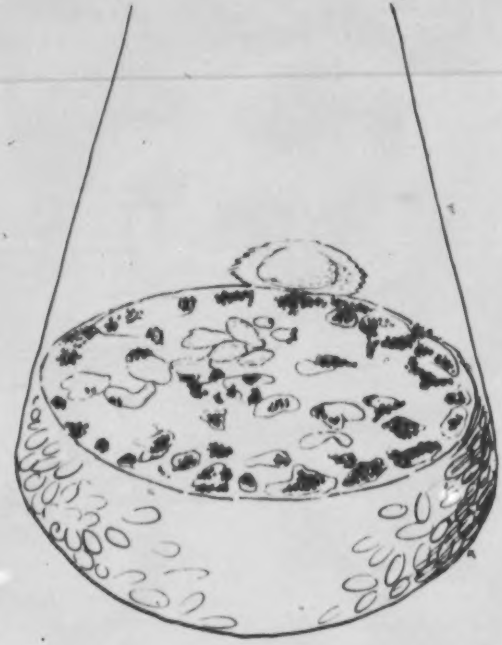

FIGURE 9 *Culture of fungus from ant nest in flask (large white mass is hyphal mass; small circles represent white hyphal aggregations).*

Solidago (Batra, 1964), and *Ficus* suggest that the fungus utilizes larval excreta as a supplemental source of nutrients and in return gives some shelter to the insect. Many larvae lie in immediate contact with these fungi in sectioned material, and mouth parts of some have actually been observed to lie in juxtaposition to hyphae, causing a dent in the latter at the point of contact.

MYCORRHIZAE

The fine feeder roots of many vascular plants are invaded by specific nonpathogenic fungi; together, root and fungus form a very distinct morphological unit, a mycorrhiza. Physiologically, mycorrhizae represent cases of symbiosis, or reciprocal parasitism, but not pathogenesis. Mycorrhizae are present on roots of most plants growing in natural sites and are more likely to be present than not. Survival of many higher plants depends upon the association of appropriate mycorrhizal fungi.

Mycorrhizae are classed into three groups on the basis of the interrelation of hyphae and root cells. The type of mycorrhiza is usually characteristic for the species of host and involves specific groups of fungi.

Types of Mycorrhizae

Ectomycorrhizae Caused by invasion of actively growing absorbing roots by hymenomycetous and possibly some ascomycetous fungi. A compact mantle of mycelium forms on the surface of the roots. Middle lamellae of epidermal and outer cortical cells are dissolved as hyphae grow intercellularly. The living epidermal and cortical cells are thus isolated by mycelial partitions. The meristematic tip and the stele are not invaded by hyphae. The infected roots are shorter than the uninfected ones, are often branched, and do not develop root hairs.

Endomycorrhizae Caused by invasion of absorbing roots by specific phycomycetous fungi. Hyphae are present on root surfaces as individual threads and penetrate directly into root hairs and other cells of the epidermis. Penetration of cells sometimes extends no farther than the epidermis; frequently, however, the hyphae grow into the cortex cells. Within the cells the hyphae may appear as coils, swellings, or minute branches. Extensions of hyphae may occur intercellularly in herbaceous plants, but usually they grow from cell to cell through the cell walls in woody plants. Hyphae do not penetrate the meristem or stele. The gross morphology of invaded roots may remain essentially unchanged, or the roots may become beaded on some species, resulting from periods of arrested growth of the roots followed by periods of active growth.

Ectendomycorrhizae Caused by invasion of feeder roots by as yet unidentified organism(s) but apparently not Hymenomycetes. They have essentially the same morphological organization as an ectotrophic mycorrhiza, but hyphae penetrate the cortical cells.

Hosts

Ecto- and Ectendomycorrhizae Betulaceae, Fagaceae, Pinaceae, Salicaceae, some Rosaceae and eucalypts. Usually only in woody species.

Endomycorrhizae Most herbaceous and woody gymnosperms and angiosperms other than those listed under ectotrophic hosts.

Associated Fungi

Ectomycorrhizal Fungi *Amanita, Boletus, Cantharellus, Cortinarius, Corticium, Lactarius, Lepiota, Paxillus, Russula, Suillus, Thelephora, Tricholoma, Rhizopogon, Scleroderma, Lycoperdon, Pisolithus, Cenococcum.*

Endomycorrhizal Fungi *Endogone, Rhizophagus*

Ectendomycorrhizal Fungi One unidentified septate fungus has been isolated.

Physiological Implications

Ectomycorrhizae have been studied more thoroughly than the endotrophic. Ecological and physiological studies on pine and beech have shown that accumulation of soluble carbohydrates in the roots of trees in some instances depends largely upon light intensity and duration, and favors secretions of a growth-stimulating metabolite into the rhizosphere. Mycorrhizal fungi, usually Hymenomycetes or Gasteromycetes, present in the rhizosphere are stimulated by the metabolite and invest actively growing short roots. Secretions of an auxinlike compound by the fungi cause the absorbing roots to develop as short, sometimes profusely branched structures. Branching is especially prominent on pines. Through secretions of pectolytic enzymes, fungi enter intercellular regions of the cortex. Restriction of hyphae to the cortex and entrance of specific fungi into roots are controlled by unknown mechanisms. Hyphae, which frequently radiate some distance into the soil adjacent to the roots, absorb nutrients from the soil and translocate them into root tissues in much greater quantities than do roots without mycorrhizae. The fungi utilize carbohydrates and probably other growth substances from the root cells. The exchange of metabolites may be altered by several environmental factors including the available nutrients in the soil and light intensity.

Endomycorrhizae have been studied primarily from a morphological standpoint. Although the physiological mechanisms involved are not well established, associations of endomycorrhizal fungi stimulate growth of at least some of the hosts. It has been observed that hyphae that invade root cells follow the advancing meristematic root tip, growing from cell to cell. In herbaceous plants, fungi may develop between outer cortex cells and send haustoria into adjacent cells. Older hyphae are digested by root cells and probably the contents are assimilated by the host. Translocation of mineral nutrients from soil to the host probably takes place as in ectomycorrhizae.

Field Collecting

Most mycorrhizae are in the top few centimeters of soil. Frequently the fine feeder roots of trees permeate moist decaying organic matter in litter and are easily exposed. This can be an excellent source of tree mycorrhizae. Late spring and early fall, when soil moisture is abundant, are preferred times for collecting mycorrhizae of trees. The best examples of mycorrhizae of most plants are found when root growth is most active.

Pinus, *Fagus*, *Quercus*, *Betula*, *Pseudotsuga*, and *Abies* have been extensively studied as hosts for ectomycorrhizae.

The fungus mantle may be seen easily with a hand lens and is white, gray, yellow, brown, pink, or black, depending upon the associated fungus.

Liriodendron, *Acer*, and *Zea* are a few of many genera that may be used to study endomycorrhizae. Superficially, the fungi may not be apparent. The root tips, however, are swollen and yellowish rather than clear white. *Acer* mycorrhizae often appear as small chains of beads and are very distinctive.

It should be remembered that mycorrhizae are formed on the smallest roots and may be only one or two millimeters in length.

The common fixative, formalin acetic alcohol (FAA), or a similar preservative, will permit mycorrhizae to be stored indefinitely. Mycelial color, however, may be lost in a few days.

Sporophores of fleshy fungi that are part of the ectomycorrhizal complex are collected from early summer into fall. Rainfall, temperature, and physiological state of the host play important roles in time of sporophore development. Host species often dictates the species of associated fungi. The occurrence of sporophores in one season does not guarantee that they will appear regularly in subsequent years. Epigeous Hymenomycetes and Gasteromycetes are easily collected and dried or preserved in liquid. Obviously they are most successfully studied when fresh. Hypogeous Gasteromycetes are found with more difficulty, since they are usually small and not readily seen.

Isolation of Fungi

Ectomycorrhizal Fungi Young sporophores are the most convenient source of sterile tissue for isolation. They offer several advantages over direct isolations from mycorrhizae: (1) The fungi can be identified by sporophore characteristics; (2) sterile tissue from within the context or stipe can be excised with reasonable ease with sterile instruments; and (3) spores can be collected and sometimes germinated.

Fungi may be isolated directly from mycorrhizae by employing techniques usual in isolation from plant tissue.

Disinfect the mycorrhizae from 10-20 min in a 1% sodium hypochlorite solution (20% commercial Clorox) or 2 min in 1:1000 $HgCl_2$ solution. Rinse thoroughly several times with sterile distilled water and plate on agar.

Ectomycorrhizal fungi are thiamin-deficient. Potato glucose agar will support growth of many species of *Amanita*, *Boletus*, *Lactarius*, *Suillus*, and probably others. The standard media used in most research on mycorrhizal fungi are: Glucose-ammonium chloride medium (M-40) and glucose-ammonium tartrate medium (M-76).

Mycorrhizal fungi grow slowly. Some of the more rapid growers are *Rhizopogon roseolus*, *Suillus luteus*, *Suillus granulatus*, and *Amanita rubescens*. *R. roseolus* requires approximately 15-18 days to cover the agar surface of a 90 mm Petri dish. Although *Russula* is a common ectomycorrhizal fungus, it is not readily cultured. Only occasionally have spores been isolated and successfully cultured.

Endomycorrhizal Fungi Most of these fungi are not easily cultured in vitro; thus inoculation studies commonly utilize sporocarps isolated from the surface of mycorrhizae. *Endogone* sporocarps may be removed from soil by wet sieving and decanting. The technique is similar to the one used by plant nematologists.

Rhizoctonia may be isolated from orchid rhizomes or roots using surface sterilization techniques. Certain strains of *Rhizoctonia solani* will stimulate germination of a number of species of orchid seeds. The endomycorrhizal fungus *Rhizoctonia* may be grown on potato glucose agar.

Morphology

Ectomycorrhizae of Pine Mycorrhizae may be sectioned with a sharp razor blade or with a microtome. Sections 12μ thick are generally most satisfactory for morphological details. Ordinary embedding procedures for plant tissues are successful. Fresh sections may be stained with phloxine B or cotton blue in lactophenol. Prepared slides can be readily stained with safranin-fast green; fungus tissue stains blue-green and host cell walls red.

The internal morphology characteristically shows no fungal penetration of stele or meristem. Hyphae form heavy partitions between outer cortex cells and the arrangement is called the Hartig net.

Endomycorrhizae of Maple Free-hand and microtome sections may be prepared in the same way as those of ectomycorrhizae.

Internally there is no fungal invasion of stele or meristem. Hyphae penetrate root hairs or other epidermal cells and are intracellular only. Coils of individual hyphae predominate over vesicles or branches. Hyphae may be digested within host cells and fragments are often visible. Host nuclei are intact and may be enlarged.

Monotropa *Monotropa* is a classical subject for mycorrhizal studies. The root system is a very compact mass of mycorrhizae. The fungal mantle completely isolates host tissue from the surrounding substrate. Mature *Monotropa* may be collected in the summer in stands of trees that have ectomycorrhizae. The fungi involved apparently are the Basidiomycetes that form mycorrhizae on trees.

Inoculations

Open Culture The most successful method is to plant host seedlings in soil from an established growing area. Pine seedlings can be grown and maintained for several months up to a year in a 5-inch clay pot. The roots form a solid mass on the inner surface of the pot, and mycorrhizae usually are very abundant. This is excellent demonstration and laboratory material for ectomycorrhizae. The root ball can be removed and replaced in the pot without destroying the source of material.

Corn or maple seedlings may be grown in the above manner. Sporocarps of *Endogone* tend to form on the surface of the root ball and are a good source of fresh material.

Pure culture The primary reason for using this technique is to establish with certainty that a particular fungus isolate will form mycorrhizae with a particular host. The procedure requires considerable time and manipulation of materials so is not necessarily recommended for general class use.

Pine seeds (preferably ones that do not require stratification) should be disinfected with 1:1000 aqueous mercuric chloride for 3 min, thoroughly washed with several rinses of sterile distilled water (H_2O_2 or Chlorox may be substituted, and do not require rinsing), and plated on agar in Petri dishes. Viability and surface sterility are easily determined with this technique. Germination usually takes 5-10 days.

In advance, fill one-liter Erlenmeyer flasks to a depth of 5 cm with sand, vermiculite, or a 1/5 mixture, by volume, of peat moss and vermiculite. The vermiculite-peat moss mixture is preferred. Moisten with approximately 275 ml of nutrient solution (M-74). The nutrient solution, before autoclaving, will have a pH of 6.5; after autoclaving, the pH will be about 4.4; replace KH_2PO_4 with the same amount of K_2HPO_4 to get final pH 5.3. For vermiculite, adjust to pH 4.0 with HCl to get pH 5.2-5.4 after autoclaving. The substrate, after autoclaving, should be moist but not wet; i.e., when tilted, neither liquid nor solid particles should move out of place appreciably.

Plug the flasks with cotton and autoclave at 121°C for 40 min. Thoroughly cool. When the seeds have germinated, the radicles not exceeding 1 cm in length, transfer one seed to a flask, carefully plant in the substrate with a sterile inoculating loop or a mechanic's "retriever," taking care not to damage the radicle. The seeds should be covered by 2 cm of the medium; otherwise seedlings tend to fall over as the hypocotyl elongates. Inoculum may be added to the flask at this time or later by introducing mycelium on agar or from solution cultures. The flasks should be maintained at a minimum illumination of 700 ft-c on 14-hour photoperiod. Mycorrhizae should be well developed within three to four months if an appropriate fungus was used. High temperatures (above 29°C) will severely restrict growth of many mycorrhizal fungi. Thus the medium within the flasks should not be permitted to exceed 29°C.

Ectomycorrhizal fungi are very sensitive to high temperatures. Several isolates have been studied, and temperatures of 32-35°C were found to inhibit growth completely. At 10° intervals from 1.7°C (35°F) to 35°C (95°F) a complete curve can be demonstrated in 24 days. Such fast-growing species as *Rhizopogon roseolus* or *Suillus luteus* may be used to illustrate this phenomenon. Both grow readily at 29.4°C (85°F), but growth is severely restricted at 32.2°C (90°F) and is prevented at 35°C (95°F). Solid or liquid media may be used; however, if liquid media are used, the mycelium must float for adequate growth.

See also Kelley, 1950; Melin, 1959; Harley, 1969; Peyronel *et al.*, 1969.

LICHENS

Collecting

Lichens are probably the easiest of all plant groups to collect. They may be collected at any time of the year, since there is comparatively little seasonal variation in size or fruiting. If collected dry, they may be preserved indefinitely without further preparation. They will not shrink or discolor.

Lichens grow nearly everywhere, on tree bark, exposed rocks, especially granite and sandstone, and on soil. Of course, the various areas in North America differ considerably in the abundance of lichen flora, but as a general rule, lichens are commonest in sparsely populated forested regions, becoming less and less so in intensively farmed regions and rare or lacking in cities or city parks. The best areas are oak forests in the Ozark-Ouachita Mountains, the Southern Appalachians, the boreal pine-spruce forests from Minnesota to New England, and high mountains in the Western States. The poorest areas are central United States, the Great Plains, and deserts.

Bark-inhabiting species are the most conspicuous and can be found from the base of the tree to the canopy branches. Specimens may be peeled from the bark with a knife or collected intact with bark. Ideally, specimens should be at least 3-6 cm in diameter. Rock-inhabiting species are usually more difficult to collect. If specimens are so closely attached that they cannot be removed with a knife, a hammer and chisel or geological hammer can be used. It is often very difficult to get suitable specimens from granite or basalt, and only practice and luck will suffice. Soil lichens grow loosely on the ground and can be easily collected, often in great quantities.

See also Fink, 1935; Llano, 1944; Nearing, 1947.

Preservation

Once a lichen specimen is obtained, it may be placed in a paper bag in the field and stored indefinitely upon returning to the laboratory. Since age does not affect the external appearance of a lichen, it is difficult to say when a lichen "dies," but ordinarily after several months of desiccation the lichen components cannot be revived in culture. If specimens are collected wet or moist in the field, they must be air dried as soon as possible, preferably within two days, in order to prevent molding. For this reason, too, polyethylene collecting bags, which have become so popular, should *never* be used for lichens.

For permanent mounting, many specimens can be put directly into standard folded paper packets, labeled, and filed vertically in shoe boxes or file drawers or pasted on standard herbarium sheets and stored in herbarium cases. However, if the specimens are bulky or do not lie flat, as is often the case with fruticose or large foliose species, the specimens should be moistened thoroughly, placed between blotters, strapped in a plant press, and dried with forced circulating air or by changing the blotters once or twice. Heat alone will usually cause mold to develop before drying is completed. When pressed, lichens lose some of their natural appearance and configuration, but they are far less likely to be damaged during handling and take up less space in the herbarium. Specimens on large branches, thick pieces of bark, or large rocks are best stored in small boxes. Neat, well-trimmed specimens form the most satisfying collection and have the greatest scientific value.

As for labels, information on locality, substrate, date, and collector are most important. Detailed habitat descriptions, color notations, or ecological notes are often desirable but not critical in identification.

Laboratory Study

Identification to genus and species is possible with one of the field guides available.

Internal structure of the thallus is best studied with one of the larger foliose forms, such as *Parmelia*. Given a dissecting microscope and a sharp razor blade, anyone can cut free-hand sections of the thallus. The sections are mounted on microscope slides in glycerine or a clearing agent (as Clearcol) so as to remove the air bubbles and render the medulla transparent. A typical cross-section under the microscope will show the dense upper cortex, an algal layer, loosely interwoven hyphae of the medulla, and a dense lower cortex with black rhizines. In general, such microscopic details as haustorial connections between fungus and alga or cytological details are difficult to observe. However, it depends somewhat on the type of lichen--species of *Lecanora* and *Lecidea* contain very large, numerous, and intrusive haustoria that are observed readily.

The ascocarps, apothecia or perithecia, may be studied in exactly the same way as those of other fungi. Spore characters, septation, color, etc., are most frequently studied. Pycnidia also occur in many species and are identical with those of other fungi. Both ascocarps and pycnidia are easily studied with free-hand sections. If a microtome is available, thin sections may be prepared with the paraffin method or with a freezing microtome. Since lichens tend to become very brittle when dehydrated, however, and considering the extra effort thereby entailed, microtome sections have little advantage over free-hand sections.

Several biological phenomena can be illustrated with lichens with a minimal amount of equipment. One of these is spore ejection. Lichen ascocarps produce spores more or less continuously over a period of several years in nature, and specimens collected at any time of the year are likely to be fertile, although very little is known about seasonal formation of spores. Spore ejection is demonstrated by selecting two or three ascocarps (preferably larger apothecial discs) from the thallus and attaching them to small corks with vaseline. The disc is moistened and the whole is placed in a Petri dish so that the surface of the discs is 2-3 mm from the inner surface of the glass. After one to three hours or longer, the glass surface above the discs will be splattered with spores, which may be observed by placing the Petri dish directly under a microscope. The maximum height of ejection is unknown for most species. In all of these experiments freshly collected specimens should be used.

Lichens produce large quantities of lichen acids that are encrusted on the hyphae. These give rise to brilliant colors in the medulla when it is treated with concentrated aqueous KOH, $Ca(ClO_2)_2$, or a 5% alcoholic solution of paraphenylenediamine. They may be extracted by placing several thallus fragments, each about 0.5 cm^2 on a microscope slide and dropping acetone over them. After the acetone evaporates, a whitish powdery or gummy residue appears. Further microchemical identification can be made with various reagents (e.g., alcohol or acetic acid) in which the residue is recrystallized as characteristic needles or prisms. Details on acid identification are given in Hale (1961).

Some of the lichen acids are known to have antibiotic properties, and standardized experiments to screen lichen species may be easily carried out. Bacteria, such as *S. aureus*, should be plated in Petri dishes and paper discs impregnated with lichen extracts added. The extent of antibacterial activity is correlated with the width of the ring around the discs that is free of bacteria. Since the majority of lichens have not been tested for antibiotic activity, this type of experiment has unlimited possibilities.

Efforts to separate the algal and fungal components and culture them separately, even to recombine the components in the laboratory and form the parent lichen again, are understandably popular but until recently only rarely successful. One difficulty is that absolutely sterile conditions must be maintained in cultures that may run several months. For this reason alone, a well equipped mycological laboratory is an absolute necessity. Many experiments that seem to succeed can often be traced to contaminants. Secondly, lichen fungi as a rule grow so very slowly that an experiment cannot be concluded in less than three to six months, and even then success may be limited. In addition, so little is known about the most suitable lichen species to use that much time can be wasted. Experiments in culturing lichen components, unless facilities are complete and the experimenter has first had considerable experience in culturing ordinary fungi, are inadvisable. For those who want to attempt it, the following guidelines will help. (See also Ahmadjian, 1967.)

- The fungal component is best isolated by germinating spores obtained by the ejection method described above. Spores may be germinated and cultured on various agars or in standard synthetic liquid media. If no visible growth occurs in one or two months, one would be advised to discontinue working with that particular species. In general, crustose species grow much faster than foliose species. Once the fungus shows signs of growing, many physiological experiments on sources of nutrients, effects of temperature, pH, etc., may be tried. It is difficult to give specific details here because so little is known about lichen fungi in pure culture.
- The algal component is isolated by dissecting bits of the algal layer from the thallus under a microscope. The fragments should be cultured on mineral agars or any other media that are unfavorable to the growth of fungi. Lichen algae also tend to grow very slowly.
- The component alga and fungus may be grown together in culture in the hope of effecting a recombination, but the optimal conditions for this experiment are very poorly known at present, and chances of success are small.

PREDACEOUS FUNGI

Included in the abundant and diverse microbial population of soil are fungi that are taxonomically distinct but ecologically a natural group, united by their adaptation to the predaceous habit. These remarkable organisms are able to capture, kill and consume microscopic animals. Their prey consists of amoebae, rotifers, nematodes, and springtails, but concern here is with nematode-trapping fungi only. Some are Phycomycetes, at least one is a Basidiomycete, but many are Fungi Imperfecti of the order Moniliales. The genera most commonly represented are *Arthrobotrys*, *Dactylaria*, *Dactylella*, and *Tricothecium*. The structures produced by these fungi for the capture of prey vary greatly in design and detail, but they trap nematodes by either adhesion or occlusion.

Some fungi capture nematodes by adhesion to virtually undifferentiated hyphae, but many have for this purpose specialized organelles that include networks of adhesive branches, stalked adhesive knobs, nonconstricting rings, and constricting rings. In spite of their morphological adaptation, nematode-trapping fungi are not obligate predators. They will grow as saprophytes, and some species have been cultivated in chemically defined media. It is of particular interest that in pure culture many of these fungi do not form traps, but in the presence of nematodes hyphae differentiate and numerous organelles of capture are produced. The presence of prey somehow induces the predator to undergo morphological change essential to the predatory relationship. There is evidence to support the possibility that nematodes produce a morphogenic substance. This substance, which was designated nemin, remains to be identified.

Nematode-trapping fungi are part of the abundant and diverse microbial population of soil. They can be detected and isolated with ease. For this purpose, one need only place a small quantity of soil, root tissue, or partially decomposed organic matter on the surface of a dilute medium such as cornmeal extract agar (M-7) and observe microscopically the sequence of organisms that develops. There will be some growth of bacteria and many common fungi will be recognized, but their numbers will be limited by the low nutrient level of the medium. Nematodes present in the inoculum will multiply, and eventually fungi that prey on nematodes will be observed. They are isolated most readily by micromanipulation of spores. Surveys have shown that predaceous fungi are extremely widespread and a method has been described for measuring the most probable number of nematode-trappers in soil. Nevertheless, a great deal remains to be accomplished before there is understanding of the ecological significance of these organisms, and no conclusion concerning the usefulness of predaceous fungi as agents for the biological control of nematode pests is possible at this time.

Pour approximately 25 ml of cornmeal extract agar into each of a series of Petri dishes. Prepare one plate for each sample to be tested. When the medium has solidified, add test materials to the center of the agar surface. Use partially decomposed wood in one plate; small bits and pieces are adequate. To other plates add freshly collected roots with rhizosphere soil attached, a bit of moss cushion, a small mound of partially decomposed grass or leaves, etc. Materials must be collected shortly before they are used or collected and stored at reduced temperature in a humid atmosphere.

Incubate all plates at 20°-28°C and examine the agar surface at weekly intervals for at least one month. Use a magnification of approximately 100X and note the sequence of organisms that develops from the various inocula. Once nematodes appear, be more painstaking in your observations. The best way to locate a predator is to search for dead prey. Examine plates for dead nematodes. When these are detected, survey the carcasses carefully for evidence of fungus hyphae and the organelles of capture produced by nematode-trapping fungi. Nematode-trapping fungi can be stained directly on the agar surface with Janus green (SR-12).

Hyphae will appear bluish-green and trapped nematodes killed by the fungus will stain bright yellow. Contrast is increased by the addition of lactophenol (SR-1), and this solution serves also to reveal hyphae located within dead prey.

See also Duddington, 1957; Pramer and Kuyama, 1963; Cooke and Godfrey, 1964; Faust and Pramer, 1964; Pramer, 1964; Eren and Pramer, 1965.

ASSOCIATIVE EFFECTS

There are many examples of inhibitory and stimulatory effects of one microorganism on another when the two are brought into association in a single culture. Of these, the spectacular stimulatory effect of *Penicillium oxalicum* and several other penicillia on sporulation of *Mycena citricolor* is a good example (Salas and Hancock, 1972). Another that works well is the effect of *Fusarium* on *Melanospora pampeana* (Heald and Pool, 1909; Asthana and Hawker, 1936; Hawker, 1939). Still another is the effect of bacteria in stimulating mushroom cap formation (Eger, 1965; Hayes *et al.*, 1969).

MYCOPARASITES

Piptocephalis

Cultures of *Piptocephalis* must be maintained on a host. Some of the best hosts for class study are *Mucor, Absidia, Choanephora*, and *Mycotypha*.

Most media that are satisfactory for host growth are also satisfactory for the parasite, but M-12, -13 are recommended. Transfer bits of mycelium or spores of *Piptocephalis* and host to agar plates and incubate near 25°C. On a highly susceptible host the long, slender, dichotomously branched conidiophores should appear about the third or fourth day. An alternative method of inoculation is to prepare a dilute suspension of a mixture of host and parasite spores and pour it over the agar plates. Germination of the parasite spores and tropism of their germ tubes toward the host hyphae may be seen after one or two days. Branched haustoria may also be observed by using the high power or oil immersion objective of the compound microscope.

To test whether *Piptocephalis* is able to grow in the absence of a host, a few spores of the parasite may be picked off and placed on yeast extract agar; the spores will germinate but the hyphae soon cease to grow.

See also Benjamin, 1959; Barnett, 1964b.

Gliocladium roseum

Gliocladium roseum can frequently be found growing on other fungi in nature or may be isolated from decaying plant material or from soil. It is a destructive parasite on numerous other fungi, overgrowing and killing many or all of the host cells. Suggested hosts are *Ceratocystis fimbriata, Trichothecium roseum, Mucor* sp., or other species with hyaline mycelium. Suggested agar medium is M-64.

Inoculate the medium with host and parasite separately about a half-inch apart. Incubate at room temperature or near 25°C. After five to seven days observe the growth of *G. roseum* over host mycelium and spores. Observations may be made easily by placing the entire plate on microscope stage and using 10X and 43X objectives. Note that there is no antibiotic effect. Cells of hyphae and spores of host are contacted and killed. Dead cells stain readily with dilute phloxine and can be distinguished from living cells. After a few days most of the cells of *C. fimbriata* will be killed.

When *G. roseum* is cultured alone on agar, two types of conidiophores develop. The *Verticillium*-type is usually formed in young cultures in high humidity, whereas the *Gliocladium*-type is often formed later.

See also Barnett and Lilly, 1962.

Gonatobotrys simplex

This fungus is parasitic on species of *Alternaria* but causes little or no harm to its host. It is not found often in nature. In culture it makes close contact with, but does not penetrate, the host hyphae. Short bulbous branches are apparently the absorptive hyphae.

Inoculate medium (M-64) with mixture of host and parasite and observe the development of mycelium, conidiophores and conidia of both. Look for contact points of absorptive branches.

By using a bit of agar on a needle, pick off conidia of *G. simplex* and place on several agar plates. To some of these plates add a drop of crude water extract of mycelium of *Alternaria tenuis* (or other species). Mycelium from several cultures in liquid medium should be combined, filtered, and washed with distilled water. It should then be finely cut in a blender and heated to near boiling in water. Filter this suspension and save the filtrate. This is the crude extract and may be sterilized by autoclaving.

After two to six days observe that *G. simplex* grows only on the plates receiving the extract. The active chemical in the extract is not known but it is effective in high dilutions.

See also Whaley and Barnett, 1963.

PLANT PATHOGENIC FUNGI

For the most part, perhaps, the study of plant pathogenic fungi has been emphasized in plant pathology departments and courses rather than in mycology as such. To some extent this is unfortunate, in view of the importance of fungi in the overall biology of the host-pathogen relationship. The mycology teacher is therefore urged to take advantage of the wealth of material contained in the *Sourcebook of Laboratory Exercises in Plant Pathology* (American Phytopathological Society, 1967). This book contains detailed information on 227 laboratory studies, a large proportion of which deal with fungi in relation to plant disease. There are short sections, also, on procurement of materials and on culture media.

On campuses where there are departments of plant pathology, the teacher of mycology can obtain much assistance and abundant materials from his colleagues in that field. The same is generally true if one has access to research installations where plant diseases are included in the program.

FUNGI PATHOGENIC TO INSECTS

The Entomophthorales constitute a small but rather distinctive order of Phycomycetes. While differing greatly with respect to habitat, the six genera appear to be closely allied structurally. Their mycelium is often much reduced, forming short, thick-walled hyphal bodies. Asexual reproduction is by means of modified sporangia (conidia), uninucleate or multinucleate, that are shot off singly from the apex of club-shaped conidiophores.

Three genera are parasitic on plants: (1) *Completoria*, with its single species, is parasitic on the prothalli of various ferns; (2) the three species of *Ancylistes*, parasitic on desmids of the genus *Closterium*; (3) at least two species of *Conidiobolus* on plant debris and others weakly parasitic or saprophytic on the pilei of *Auricularia*, *Hypochnus*, and other Basidiomycetes.

Basidiobolus, though wholly saprophytic, is noteworthy because of its unusual developmental cycle that, at one stage, involves insects. The species *B. ranarum* grows on the excrement of frogs and lizards. The sporangia produced on this substrate are eaten by beetles (principally Carabidae, Scarabaedae, and Silphidae) that prowl the excrement; these beetles, in turn, may be devoured by frogs. During digestion within the frog's stomach, the spores are delimited within the sporangia and are set free. The spores, if retained for a long time, may then multiply further by division or by budding. They remain dormant in this state until excreted with the feces; once exposed to the outer air, they germinate to form mycelium on which are produced other sporangia.

The remaining genera, *Entomophthora (+Empusa)* and *Massospora*, are true entomogenous fungi that develop in the bodies of immature or adult stages of various insects. The former includes the vast majority of known species of the order.

Occurrence

Insects infected with the more common *Entomophthora* species are sometimes accidentally found in areas within easy reach of a country district or a wooded area. Many hosts before death seek conspicuous positions by crawling upwards on grass or other substances. Perhaps the favorite position is under the bark of trees, the underside of leaves in shady situations, or about barns or houses, where careful search during wet weather seldom fails to disclose numerous specimens. However, the most productive source of *Entomophthora* species consists of the numerous naturally infected insects either in collections sent directly to specialists for identification or from those obtained through insect surveys. The objective of many insect surveys is primarily to follow the population level and spread of one or more insects, to detect insect outbreaks, and as an annual census of insect conditions generally. In the course of these field observations, epidemics are sometimes noted; more frequently a number of dead insects may be found within an insect cluster, and occasionally living specimens.

Insects are considered as infected by fungi when they show indications of mycelial growth on the surface or are hard and mummified. Some degree of success has attended attempts to culture these forms and it now seems probable that many will be found to develop saprophytically on the proper nutrient media. Most attempts to cultivate *Entomophthora* species in vitro have involved the use of various kinds of complex natural media, including swordfish, pork, and others rich in protein (Sawyer, 1929). An egg medium described by Müller-Kögler (1959) that supports the growth of a number of *Entomophthora* species is one of the best media that has been developed so far. In the preparation of this medium the yolk is separated from the white and shell (previously washed and sterilized in a 70% ethyl alcohol - 1% acetone solution) under aseptic conditions. Five ml of egg yolk is placed in a sterilized tube, the tube is plugged, and the yolk then coagulated by heating at 80°C for 40-50 min in a slanted position.

Various techniques have been suggested with the object of suppressing contamination, but in recent years media containing the fluorescent dye rose bengal and antibiotics have been used increasingly in the isolation of fungi. A commercial sodium hypochlorite bleach diluted to contain 2.5% available chlorine is now used routinely in the surface sterilization of fungus-infected insects. To this preparation penicillin, 139 mg, and streptomycin, 200 mg/100 ml are added. The solution is particularly effective in the isolation of *Entomophthora* species in pure form from diseased aphids.

Freshly thrown off conidia, when required for inoculum, are collected on a sterile glass slide supported 2-3 mm above a freshly-dead infected insect placed in a dry sterile Petri dish. The conidia are then used within 12-24 hours, as a longer waiting period may lead to decreased viability and increase the danger of contamination by saprophytic fungi. To inoculate the nutrient tubes, a small portion of the medium is removed with an inoculating needle, rubbed over the collecting surface of the slide, and then replaced on the medium.

Preparation of Inoculum

Success in infecting insects with an entomogenous fungus depends largely on: (1) Obtaining a pure culture of a fungal isolate, virulent for a given insect host; (2) positioning the infective stage on or within the host; and (3) duplicating the environmental conditions that are conducive to infection.

Cultures of *Entomophthora coronata* (= *Delacroixia coronata*) and *Beauveria bassiana* can be carried on on Sabouraud's glucose agar (M-47) containing 0.2% yeast extract. If maintained in a stock culture at 9°C, they should be transferred every three months.

To prepare inoculum of either fungus, inoculate Petri dishes (150 x 20 mm) containing Sabouraud's glucose agar and incubate for 5-7 days at 26°C. Again using aseptic techniques, cut discs of the active growing culture with a sterilized cylinder (four cuts with a 2-cm cork borer) and transfer these to a sterile Waring blender. Add 100 ml of sterile deionized water and homogenize for 20 sec at low speed. Transfer 1 ml of the homogenized fungal suspension into each of several Petri dishes containing Sabouraud's agar, slowly rotating the dish to insure maximum coverage, and incubate at 26°C. This preliminary technique may be applied in the preparation of both *E. coronata* and *B. bassiana.*

Infecting Termites

The eastern subterranean termite, *Reticulitermes flavipes*, can often be obtained from trunks of fallen trees or old decaying fence posts. Sections of this infested material may be brought into the laboratory and stored in large 25-gal galvanized containers, at room temperature. Moist paper towels should be periodically added to the container to assure high humidity. Several wooden fir blocks 1" x 6" x 2", each containing a single 1/8" groove, may be held together with rubber bands and placed adjacent to the infested log (Osmun, 1957). Termite colonies will soon become established within these blocks. Recovery of termites can be accomplished simply by separating the wooden pieces and tapping one end slightly. Watchmaker's forceps that have a small quantity of solder on one of the interfaces to prevent complete closure can be used in handling termites without injuring them.

Invert Petri dishes containing 24-48 hour cultures of *E. coronata* and insert into the inverted lid a pre-cut filter paper that has been slightly dampened with sterile water. Introduce 20 adult termites into each of the inverted inoculating dishes and hold under constant light at 26°C. This arrangement will insure that the termites receive an abundance of infectious conidia and yet are not entrapped by the sporulating fungal culture. After 12 hours, transfer the termites to another Petri dish in which a 4-cm disc of 4% water agar was attached to the lid above the termites. This will insure a relatively high humidity, while the new dampened filter paper will serve as a food source.

After four to five days, remove both live and dead specimens and surface sterilize in 5.25% sodium hypochlorite for 3 min, and subsequently rinse several times in sterile water. The surface-sterilized specimens can then be individually and aseptically affixed to the inside surface of a Petri dish lid by a small quantity of white petroleum jelly. If the termites were susceptible to the fungus and are infected, conidiophores originating from the actively growing fungus in the body cavity will drop conidia on the nutrient medium. After a sufficient number of these conidia are released onto the medium below, the lid and attached specimen may be removed and replaced with a sterilized lid. An examination of the developing pure culture should confirm the identity of the fungus. The culture can then be used to reinoculate other termites as above, if one wishes further to confirm its pathogenecity.

It must be recognized that the mere isolation of a microorganism from a dead insect does not, of itself, demonstrate a pathological relationship.

To evaluate the pathogenicity of a given isolate against termites, prepare the inoculum as previously described and dispense 1 ml of the suspension into each of five Petri dishes. Prepare a like number of Petri dishes as a control that are not inoculated with the fungus suspension. After the appropriate time for incubation, place 20 termites in each Petri dish--this gives a total of 100 termites inoculated and 100 in the control.

Observations should be made 2-3 days after inoculation and the percent mortality calculated. Routine autopsies should be conducted by preparing squash mounts of dead termites. This may be accomplished by placing the termite to be autopsied between a glass slide and the cover slip and pressing firmly on the cover slip. Observations can then be made with a compound microscope to determine whether the fungus has developed extensively within the body cavity of the termite. Mortality data can then be critically interpreted. To study the histopathology of *E. coronata*, inoculate termites as previously described. Treated and untreated termites should then be removed and placed in a fixative at two-hour intervals for the first 48 hours and every 3 hours for the remaining 24 hours. For comparison of noninfected specimens, termites can be treated similarly except that the inoculum is omitted. When these specimens have been prepared for histological examination, the course of the mycosis can be followed in detail from the time the germ tube penetrates the cuticle to the entire destruction of virtually all internal tissue.

Infected termites show progressive symptoms of sluggishness, uncoordinated locomotion, and, when placed on the dorsal surface, are unable to right themselves. In pathogenicity tests, 50% or more mortality should occur within 36 hours after the termites are inoculated with *E. coronata*. The total mean mortality after some 84 hours should be approximately 95%-98%.

Histopathological observations show that integumental penetration of the termite is not restricted to any specific region of the body wall and will often occur within 12 hours after inoculation. It will further be observed that germinated conidia ingested by the termite will penetrate the fore intestines and then grow abundantly within the body cavity. Upon penetration, the mycelium spreads rapidly within the body cavity of the termite and maintains its filamentous structure. After much of the tissue has been destroyed, cell walls are produced in the mycelium. The resulting structures then take on the form of compact hyphal segments. Death as a result of the mycosis usually occurs as early as 12 hours after inoculation, the mortality rate increasing greatly after 32 hours.

Wax Moth Infection--Entomophthora

Starts for a wax moth culture can usually be obtained from an apiculturist or from a reliable bait store that sells "bee moths" for fish bait. Larvae are easily reared on a diet composed of: Honey, 100 ml; glycerine, 100 ml; water, 50 ml; pablum mixed cereal, 1000 ml; Kellogg's Special K, 250 ml. The diet can be prepared and placed in quart or larger fruit jars. Larvae are then introduced into the medium and the jars capped with fine-mesh copper or plastic screen in order to prevent newly hatched larvae from escaping. The screen is held in position by the metal screw ring.

When the wax moths are obtained from an apiculturist, they are usually in the pupal stage. These may be placed directly in a jar without medium. The adults will soon emerge, copulate and deposit their eggs. The small larvae upon hatching can then be placed on the prepared diet. As the larvae mature, they will spin a cocoon. Strips of corrugated paper placed in the jar provide an ideal area for these cocoons, which can then be removed and placed in a new jar when desired. The length of life from hatching to adult emergence at room temperature will usually average 30 days.

To inoculate wax moths with *Entomophthora*, prepare the inoculum as previously described and incubate for 4-5 days. Following incubation place five larvae of the greater wax moth, preferably those in the later instars, into the Petri dish containing the inoculated medium. Allow the larvae to crawl on the surface of the culture in each dish for 15 min. Transfer these larvae to a sterile Petri dish containing a moistened filter paper, which will ensure optimum humidity for conidium germination and subsequent penetration of the wax moth larvae. Control larvae should be treated in a similar manner except the inoculum is omitted. This test can be conducted at room temperature.

To assay infectivity, inoculate wax moth larvae by the above method and examine every 24 hours. Controls should be treated in the same way. Various fungal isolates may also be tested as to their virulence in this manner. It is recommended that 100 wax moth larvae be used for each treatment. Use 5 or 10 larvae in each treatment and replicate to give a total of 100 larvae per treatment. On the basis of these data the percent mortality can be calculated. During each observation remove dead larvae from the Petri dish and place for three minutes in 5.25% sodium hypochlorite to surface-sterilize the specimens. Larvae should then be rinsed in sterile distilled water and placed on nutrient medium. Within 48 hours hyphae will emerge from the larvae that are killed by the fungus and grow abundantly on the medium.

For histological studies, sequentially sample two or three of the inoculated larvae every 6 hours for a period of 3-4 days. These larvae should be fixed and prepared for histological examination. The infection process can then be followed by observing a series of these prepared sections, i.e., the penetration of the germ tube, the change in fungal morphology once inside the body cavity of the insect, the general destruction of the internal organs and the post-mortem condition of the insect.

Sectioned material should show that the fungus is capable of passing directly through the thick sclerotized body wall, apparently by producing a substance capable of breaking down the body wall. There is some reason to believe that a mechanical action is also involved.

After penetrating the body wall, the fungus will attack the fat cells. The other organs, i.e., silk glands, intestines and malpighian tubules, are also destroyed. Most investigators find that the last structures to be invaded are muscles, nervous system and gonads.

Some wax moth larvae infected with *E. coronata* will die the first day after inoculation, but most will succumb on the second post-infection day of the experiment. Larvae that are infected will soon become weakened and flaccid. The mobility of moribund larvae is usually impaired. Black spots may often form on the integument of the infected host.

Larvae that have been killed by the fungus, if placed in a suitable environment of high humidity (e.g., on nutrient agar) will produce conidiophores abundantly. These conidiophores originate from the "hyphal bodies" within the host and develop conidia within 20 hours. "Hyphal bodies" are generally found throughout the body cavity of the larvae and will appear in longitudinal sections made of larvae in the advanced stages of infection. After death of the host, the mycelium can be found throughout the entire body, attacking virtually all internal tissue.

In pathogenicity tests, mortality is variable, ranging from 50% to 80%, depending upon the isolate.

Wax Moth Infection--Beauveria

Infectious material of *B. bassiana* can be prepared as described above except that a longer incubation period is required before introducing larvae. Four or five days should be sufficient to develop an abundant source of conidia. After the incubation period introduce larvae into the Petri dishes and allow them to crawl over the culture for 4 hours. The remaining techniques used for *E. coronata* can also be applied in the study of the infectious process of *B. bassiana*.

Infected larvae become rather sluggish and do not respond readily to external stimuli. At times, depending solely on the particular isolate of *B. bassiana* used, a characteristic symptom of the disease in the larvae will be a change in color ranging from a white to pinkish cast. As the infection progresses, the larvae become soft and pliable until the mycelium has ramified and spread throughout the body cavity and internal tissue. The body soon becomes rigid and finally mummified.

See also Dresner, 1949; Wolf, 1951; Smith, 1953; MacLeod, 1954a; Doane, 1959; Prasertphon, 1963; Yendol and Paschke, 1965.

MEDICAL MYCOLOGY

SPECIAL PRECAUTIONS

Because the fungi included in the following section are capable of infecting human beings, they should be handled with the utmost care and in no instance distributed to students unless the instructor and the students are entirely familiar with the precautions needed to make the risk minimal. Systemic pathogens should, under no conditions, be used as live cultures in the general mycology laboratory.

All fungus cultures should be grown on slants of Sabouraud's glucose agar (M-47), with the exception of those that require special media, such as the yeast phases of the diphasic systemic fungi and species of *Actinomyces*. Sabouraud's glucose agar is used in many laboratories throughout the world and serves as a standard reference medium. In addition, special media can be used to stimulate sporulation or to demonstrate certain morphological characteristics. Thus species of *Candida* and the causal agents of chromoblastomycosis are maintained on Sabouraud's and also on cornmeal agar in order to demonstrate characteristic sporulation.

All cultures can be studied in a viable state except the mycelial phase of *Histoplasma capsulatum* and *Coccidioides immitis*, which are highly infectious. These cultures should be treated with formalin before being distributed. The other deep or systemic mycoses should be handled with *special care*, using sterile techniques at all times. A safety hood should be available for transfers.

Clinical Materials for Study

Clinical materials may be either obtained from hospitals or clinics or prepared by the inoculation of experimental animals or by artificial seeding of body fluids. Materials that may be used are listed below under the different disease categories:

Tinea versicolor Skin scrapings from a few patients will give ample material that can be saved for many years. The material may be kept in a small specimen bottle with a cap or cork.

Ringworm of the skin and nails Skin scrapings and nail clippings can be obtained from a hospital clinic or dermatologist's office for use in culturing and in KOH mounts.

Ringworm of the scalp Characteristic *Microsporum*-infected hairs may be obtained from a case in the hospital clinic or from a kitten or puppy in the veterinary clinic. Infected hairs may be produced experimentally by inoculating guinea pigs with *M. canis* and collecting fluorescent hairs after about three weeks. Ectothrix *Trichophyton* hairs are easily produced by inoculating guinea pigs with granular cultures of *T. mentagrophytes* and collecting infected hairs after 10-21 days.

Systemic mycoses The following systemic mycoses may be readily produced in experimental animals, thus providing ample materials for study: candidiasis, coccidiodomycosis, cryptococcosis, histoplasmosis, North American blastomycosis, sporotrichosis, and nocardiosis. Appropriate methods for producing these infections are found in any laboratory manual on medical mycology. Granulomatous material from the peritoneal cavity of mice infected with *Coccidioides immitis* or *Blastomyces dermatitidis* may be kept in lactophenol for later use to mount as small portions in a drop of lactophenol on a glass slide with a cover slip.

Human sputa may be collected from a diagnostic laboratory, autoclaved, and pooled (or normal human sputa may be used). This may then be seeded with yeast phase cells of *Histoplasma capsulatum*, *Blastomyces dermatitidis*, or *Paracoccidioides brasiliensis*. If the sputum is refrigerated, the organism may remain viable for several weeks and the material is useful not only for demonstration of the organism but for cultural studies.

To demonstrate the characteristic granules of Actinomycosis, infected cow's jaws may be obtained from local slaughterhouses. A stock of *Actinomyces* granules may be obtained by collecting them from infected animals and storing in 10% formalin.

Maintenance of Stock Cultures

There are several ways to maintain culture collections of pathogenic fungi. With the exception of lyophilization, all methods are easy to carry out without special equipment.

Room Temperature Stock cultures are maintained in racks or baskets for up to three months before subculturing. The length of time varies, depending upon the type of plug, relative humidity and the size of the test tube, before the medium dries out and the culture dies. A sealed or capped tube will not dry out as rapidly as unsealed ones.

Refrigeration Stock cultures may be maintained for up to four months before transferring to new slants. Capped tubes or sealed tubes may usually be kept for six months. Some of the *Trichophyton* species that do not produce microconidia readily, *Epidermophyton floccosum*, and *Microsporum audouini* should be maintained at room temperature, as they usually die when refrigerated.

Deep Freeze Stock cultures that are a week and a half old are placed in the deep freeze until needed. Screw-capped tubes should be tightly closed before being placed in the deep freeze. Viable colonies may be maintained for at least two years by this method. Sterile litmus milk may be added before tubes are frozen.

Mineral Oil Stock cultures can be maintained by pouring sterile mineral oil (Saybolt viscosity 330 at 100°F) over the entire surface of an agar slant bearing an actively growing colony. The cultures may be viable for several years when kept in an upright position. When it later becomes necessary to make a transfer, a small portion of the fungus is removed from below the surface of the mineral oil and, after excess oil drains off, is placed in a new agar slant.

Lyophilization This method is excellent for certain types of fungi and will preserve them in a stable condition for an indefinite number of years. Many small-spored fungi such as *Penicillium*, *Aspergillus*, *Actinomyces*, and some of the yeasts, as well as other microorganisms, have been preserved by this method. Many of the poorly sporulating pathogenic fungi or large-spored forms are not very suitable for lyophilization.

Killed Cultures Pathogenic fungi in bottles or tubes may be killed by dipping cotton plugs into 40% formalin and inserting or reinserting them. The opening of the container should be sealed to prevent drying out. Pigments and the appearance of the colonies deteriorate over a period of time.

Laboratory Study

A number of the following exercises may be carried out individually or as a group demonstration to illustrate certain laboratory techniques.

Staining There are a number of special staining techniques used especially for demonstrating fungi in tissues, such as periodic-acid-Schiff stain, Gomori's methenamine-silver nitrate technique, or Gridley's histological stain. More detailed information may be found in standard references.

Isolation of Dermatophytes from Soil Many of the fungi pathogenic to man and animals have been found in recent years as saprophytes in soil or decaying plant material.

These organisms have been isolated on agar media, with or without the addition of antibiotics, by the hair-baiting technique or by injecting the supernatant from the soil samples into mice in cases wherein systemic fungi were being investigated.

Samples of soil and other materials may be collected in small bottles or plastic bags for laboratory study. Types of habitats where pathogenic fungi have been isolated are: Animal burrows; animal carcasses; basement soil; soil inside or adjacent to chicken houses; soil from dog pens; soil from feedlots; soil from garages; soil from gardens and yards; animal and human manure; soil from tobacco barns and beds; and decaying wood and vegetation.

- Fill sterile Petri dishes half full with soil sample. Add 15-20 ml of sterile distilled water to the soil if it is needed to make the soil moist.
- Scatter short strands of sterilized human or horse hair over the surface of the soil. If small clear areas are made, and the hair extends over the clearings in the Petri dish, a search may be made in a few weeks directly under the low power objective of the microscope for evidence of developing hyphae along the hair shaft.
- Incubate the preparations at room temperature (20°-25°C) in the dark for periods up to six weeks.
- Examine the hairs periodically for the development of mycelium on the sides of the filament.
- Remove hairs that show fungus growth and place in tubes or plates of Sabouraud agar containing cycloheximide and chloroamphenicol (0.5 and 0.05 g/liter, respectively).
- After one or more weeks, check the colonies and try to identify the genus and species. Pure cultures may then be made by transferring single conidia to new media.

Types of fungi that may be isolated by the hair-baiting technique include:

Microsporum gypseum complex--pathogenic in animals and man (perfect stages, *Nannizzia gypsea* and *N. incurvata*)

Microsporum cookei--(perfect stage, *Nannizzia cajetani*)

Microsporum nanum--pathogenic in animals (perfect stage, *Nannizzia obtusa*)

Trichophyton ajelloi--saprophyte, rare on animals (perfect stage, *Arthroderma uncinatum*)

Trichophyton terrestre complex--saprophyte (perfect stages, *Arthroderma lenticularum* and *A. quadrifidum*)

Perfect Forms of Some Dermatophytes The soil-hair culture technique may be used to study the perfect stage of certain dermatophytes. Several of the dermatophytes will produce cleistothecia under suitable cultural conditions. The perfect stages of two genera, *Nannizzia* and *Arthroderma*, can be grown by using this technique. A Beech-nut baby oatmeal agar medium developed by Weitzman and Silva-Hutner (1967) is very useful for producing fertile cleistothecia for mating studies. It may be used after the strains are isolated from soil.

Two compatible strains for each species are inoculated on the surface of soil (sterilized or unsterilized) or oatmeal agar in close proximity. Sterilized horse mane or tail hair, or child's hair, cut short, should be scattered over the surface of the soil. Human hair is unsatisfactory for the best production of cleistothecia (Dawson *et al.*, 1964). In approximately three weeks mature cleistothecia should be developed. One may, for example, start with two strains of the heterothallic *Microsporum gypseum* and inoculate these in close proximity on the soil-hair culture medium or oatmeal agar and incubate at room temperature (24°C) for three or four weeks. Where the two strains unite, cleistothecia of *Nannizzia incurvata* will appear. Examine for presence of asci and ascospores and peridial hyphae (Stockdale, 1963, contains more details). Crosses can be conveniently carried out on the oatmeal agar developed by Weitzman and Silva-Hutner.

Examination of Skin or Nails Skin scrapings or pieces of nail material from clinics or from members of the class should be placed in a clean container, plastic bags or other suitable envelopes, using sterile technique when handling the material. The infected material may be cultured directly on Sabouraud's glucose agar with antibiotics (M-44).

- Place small pieces of infected skin or nail material on a slide containing a drop of 10%-20% KOH and add a cover slip. The addition of a small amount of Superchrome ink or other dyes may aid in differentiation of the fungal hyphae. Heat gently to clear and soften the material.

- Microscopically, the fungus appears as septate, branching mycelial strands growing through the host cells. The threadlike hyphae of the fungus may be seen under the low power of the microscope (10X) but more details of the hyphae and arthrospore formation may be seen under higher magnification. (Do not mistake fat globules or air bubbles for arthrospores or the crystals of potassium hydroxide for the branching mycelium of the fungus.)
- Try culturing any positive material that remains in the sterile container. After one or two weeks, check the culture for morphological characteristics. Make a slide mount in lactophenol cotton blue, using sterile technique, and examine for presence of spores. Try identifying to genus and species from reference books on medical mycology. Sterilize colony before discarding.

Colony Characteristics One of the fundamental bases for identification of a dermatophyte is the macroscopic appearance of the colony. A standard medium such as Sabouraud's glucose agar usually provides the basis for for the description in the literature. Cultures that are likely to be available locally for study are: *Microsporum gypseum* (from the soil-hair baiting technique), *Microsporum canis* (from a cat or dog), *Trichophyton mentagrophytes* or *T. rubrum* (from a human ringworm case). Other cultures may be ordered from culture collections or from institutions where medical mycology is taught.

- Study the surface texture, color, folds, rate of growth, and the color on the reverse side of the colony for each organism.
- Microscopic characteristics are important in most cases. The large septate macroconidia of each genus and species (when produced) vary in size, shape, and wall structure. The microconidia are small, oval or pear-shaped, borne along the sides of the hyphae or in grape-like clusters on ends of branches, and are not distinctive for species or genus. Other structures may be present, including chlamydospores, spirals, etc., which are non-specific.

- Colonies of the various pathogenic fungi studied may be grown on Sabourbaud's in large tubes or in large capped bottles to observe the development of characteristic colonies. Ordinarily, about two weeks at room temperature (24°C) is required for the development of typical colony characteristics. Check reference books for more detailed descriptions of various dermatophytes and fungi causing the systemic mycoses.

Culture of Dimorphic Fungi Many of the subcutaneous and deep mycoses regularly produce a yeast-like phase in tissue at 37°C in contrast to a mycelial phase at 24°C. Fungi causing South American blastomycosis, North American blastomycosis, coccidioidomycosis, histoplasmosis, candidiasis, sporotrichosis, and chromoblastomycosis are examples of this phenomenon. These organisms can be grown on Sabouraud's glucose agar at 24°C. *(Special care should be observed in transfer of cultures of Coccidioides immitis and Histoplasma capsulatum, as the spores are highly infectious.)* Organisms causing blastomycosis, histoplasmosis, and sporotrichosis can be converted to the yeast phase on brain heart infusion agar or other suitable media at 37°C. *Candida albicans*, the usual cause of candidiasis, produces budding yeast cells on most media, but on cornmeal agar or other chlamydospore agars at 37°C will produce pseudohyphae and chlamydospores. Any of these organisms can be inoculated into mice for the tissue phase. For more detailed information, see laboratory manuals or textbooks.

Animal Inoculations This technique is of value to demonstrate gross lesions and to provide the students with material for fresh and stained mounts that demonstrate the appearance of the organism in tissue. Information on inoculation of animals with a specific pathogenic fungus is usually listed under procedures for that organism in references on medical mycology. Organisms that may be used for animal inoculations with less concern than other mycoses are: *Sporothrix schenckii* or *Candida albicans*. *Candida albicans* occurs frequently in humans in the mouth, skin, intestinal tract or vagina of many normal individuals or may produce a diseased condition under modified conditions. The organism may be injected as a 1 ml suspension intraperitoneally into white mice. In 7-10 days the kidneys should have white abcesses. Gram-stain smears of the kidney material should show the pseudohyphae and budding yeast cells.

Other systemic mycoses may be established in mice. For more details, reference should be made to laboratory manuals or textbooks.

Biochemical Tests Some species of *Trichophyton* are readily separated on the basis of biochemical requirements (Georg and Camp, 1957). The following procedure may be used for preparation of the media for inoculation of *Trichophyton*. The media are available commercially.

Acid-cleaned glassware should be used for the nutritional tests. The stock media consist of casamino acids glucose agar (M-34) or ammonium nitrate agar (M-73), while the test media have added thiamine (thiamine hydrochloride, 0.2 μg/ml of medium), inositol (inositol, 50 μg/ml), nicotinic acid (2 μg/ml), or histidine (30 μg/ml) singly or in combination.

In preparing stock solutions, use the following amounts: Histidine solution - 150 mg/100 ml distilled water; inositol solution - 250 mg/100 ml distilled water; nicotinic acid solution - 10 mg/100 ml distilled water; thiamine solution - 10 mg/1000 ml distilled water at a pH of 4-5. Sterilize each for 10 min and store at 5°C. Two ml of the above stock solutions of vitamins will give the correct concentration in the media for the differentiation of *Trichophyton* species.

The inoculum may be taken from cultures grown on any of the usual media. It is important to take a very small, uniform fragment for each transfer to avoid a carry-over of the nonvitamin-free medium. In reading the results on Table 5 for the nutritional patterns, a 4+ indicates maximum growth for the series of test tubes when compared with the colony growth of other tubes. A ± indicates a trace of submerged growth around the inoculum. The use of these media is especially helpful in differentiation of *T. mentagrophytes* from *T. equinum*, *T. tonsurans* from *T. mentagrophytes*, *T. gallinae* from *T. megninii*, and *T. verrucosum* from some strains of *T. schoenleinii*.

TABLE 5 Nutritional Patterns for *Trichophyton*

Species	Test Media	
	Casamino Acids	Casamino Acids + Nicotinic Acid
*T. equinum**	0	4+
*T. mentagrophytes**	4+	4+

	Casamino Acids	Casamino Acids + Thiamine
*T. mentagrophytes**	4+	4+
*T. rubrum**	4+	4+
*T. tonsurans**	± to 1+	4+
*T. violaceum**	±	4+
*T. ferrugineum**	4+	4+

	NH_4NO_3	NH_4NO_3 + Histidine
*T. gallinae**	4+	4+
*T. megninii**	0	4+

		Casamino Acids	Casamino Acids + Inositol	Casamino Acids + Thiamine	Casamino Acids + Thiamine + Inositol
*T. schoenleinii***		4+	4+	4+	4+
*T. verrucosum***	84%	0	±	0	4+
	16%	0	0	4+	4+
*T. concentricum***	50%	4+	4+	4+	4+
	50%	2+	2+	4+	4+

*Use two media at room temperature, 7-10 days.
**Use four media at 37°C, 7-14 days.

See also Ainsworth, 1952; Ainsworth and Austwick, 1959; Ajello *et al.*, 1963; Haley, 1964; Emmons *et al.*, 1970; Hazen *et al.*, 1970; Beneke and Rogers, 1971; Conant *et al.*, 1971.

20 Ecological Sites

FUNGI IN INDUSTRY

Generally, laboratory work in the general area of study that might properly be called "industrial mycology" requires rather more complex equipment and wider experience on the part of students than is to be found in most introductory mycology courses. For the most part, work of this kind belongs in a second level, specialized course in physiology of fungi. A few exercises are included here to suggest the scope of "industrial" work and to assist instructors who have a particular interest in this aspect of the subject.

Measurement of Fungus Growth

Three methods for the measurement of growth of fungus mycelium are in common usage. Any one may be appropriate in certain types of investigations, but the choice will have to be made on the basis of the degree of precision sought. Mycelial growth may be estimated by: (1) Measuring diameters of fungus colonies growing on solid media in Petri dishes; (2) measuring the rate of advance of the mycelial front over a narrow strip of agar; or (3) measuring the dry weight of mycelium cultured in liquid medium. To compare these:

- Prepare six Petri dishes, one pair of which contains

0.5% KH_2PO_4, 2% glucose and 2% agar; a second pair should contain 0.5% KH_2PO_4, 2% glucose, 0.1% Difco yeast extract, and 2% agar; the third pair should contain 0.5% KH_2PO_4, 2% glucose, 0.7% Difco yeast extract, and 2% agar.

- Prepare three long tubes, each one of which contains about 8 ml of one of the three types of media listed above.
- Prepare 50 ml of each of the three types of media listed above, but omit the agar and place each of the three portions in a separate 250-ml Erlenmeyer flask.

After the media have been sterilized and cooled, inoculate the tubes, plates and flasks with *Myrothecium verrucosum, Chaetomium globosum, Colletotrichum trifolii, Sordaria humana, Alternaria* sp.

Observe the Petri dish and long tube culture after 24 hours. Measure the diameter of a colony and then measure at right angles to the first diameter; average these measurements and record. Repeat at 24-hour intervals for three or four days.

Mark the mycelial frontier in each long tube with a glass-marking pencil; repeat at 24-hour intervals for three or four days.

When obvious mycelial mats are formed in the flask cultures, harvest the mats by filtering each one on a weighed filter paper. Dry at 95°C and weigh.

Temperature and Rate of Respiration

That respiratory rate is affected by temperature is well-known. To demonstrate this a simple type of respirometer can be constructed readily from supplies common in all laboratories. By substituting glass for rubber in the gas system and by maintaining accurate temperature control, a respirometer of this type will prove to be highly accurate. It has an advantage over many respirometers in that changes in gas volume are read directly in milliliters without the necessity of calibrating and establishing a factor for the respirometer.

Since this exercise extends over a relatively short time period, the following medium, if freshly prepared, can be used without being sterilized: Difco yeast extract, 1.4 g; KH_2PO_4, 1 g; glucose 30 g; and water, 200 ml.

Operation of Respirometer Prior to starting the apparatus (Figure 10), the mercury level in pipette *B* should be raised to *0* (indicated by arrow). This operation should be performed with stop-cocks *E* and *F* open so that the liquid levels remain the same in both arms of manometer *A*. Then close stopcock *E*.

- Fill respiration chamber *D* with medium to within about 1/2" of the stopper and inoculate.
- Close *F* for 5-10 min (shorter or longer periods may be used depending upon the type of yeast and size of inoculum). As CO_2 is evolved from the respiration chamber, the liquid level in the left arm of manometer *A* is depressed.

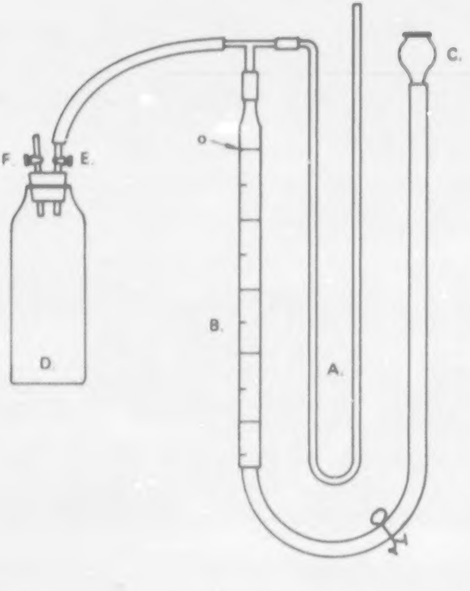

FIGURE 10 *Diagram of respirometer.*

- For making a reading, *E* is closed and *F* opened. By means of the leveling bulb *C*, mercury is withdrawn from 10-ml measuring pipette *B* until the liquid levels in *A* are at the same height. The volume of CO_2 evolved is equal to the volume of mercury withdrawn from the system, which is read directly on pipette *B*.

Experimental Procedure To test the effect of temperature, mix about one-fourth of a cake of Fleishman's yeast in 15-20 ml of medium and pour into the respiration chamber; add a quantity of medium sufficient to nearly fill the chamber. After 5-10 min, connect chamber with the manometer system and obtain a reading. State respiration rate as milliliters of CO_2/min. Set the respiration chamber in ice water for 5-10 min and obtain another reading. Repeat, but set the respiration chamber in water at a temperature of 32°-35°C.

Aeration and Respiration

This exercise not only illustrates the effect of aeration upon an anaerobic process such as alcoholic fermentation but also provides opportunity to learn the use of a refractometer.

- Prepare the following medium: KH_2PO_4, 3 g; Difco yeast extract, 4.2 g; glucose, 90 g; water, 600 ml.
- Place 10 ml portions in each of three 50-ml Erlenmeyer flasks. Divide the remainder into three equal portions; place one portion in a 500-ml Erlenmeyer flask, a second in a 1000-ml Erlenmeyer flask, and a third in a 2000-ml Erlenmeyer flask. Stopper with cotton and autoclave. Measure the diameter of the liquid surface in the large flasks and calculate the surface area in square centimeters. The surface/volume ratio can thus be calculated for each flask, thus providing some indication of the degree of aeration.
- Inoculate the 10-ml portions from a slant of *Saccharomyces cerevisiae*. Incubate for 24-36 hours at 30°C.
- Inoculate each of the three large portions with one of the 10 ml cultures and incubate for 72-96 hours.
- At the end of the incubation period, remove a 50-ml

sample from each flask and distill each into a 50-ml volumetric flask (distill over about 25 ml and then bring to volume with distilled water).

• Determine the total amount of alcohol produced in each culture by means of the Bausch & Lomb immersion refractometer. The results obtained with this instrument have a high degree of accuracy if the instrument is properly manipulated; however, the refractometer prism may be damaged very easily and must be used with care.

Inoculum Size and Fermentation Rate

It has long been recognized in industrial fermentations that, within certain limits, the larger the inoculum the faster the fermentation rate.

• Prepare a sufficient quantity of the following medium so that there will be a 100-ml portion for each individual participating: KH_2PO_4, 0.5 g; Difco yeast extract, 0.7 g; glucose, 15 g; water, 100 ml. Place 100-ml portions in 500-ml Florence flasks plugged with cotton and autoclave.

• Each flask should be inoculated with 10 ml of a yeast suspension, the cell count of which is different from that used in other flasks. The following cell counts are suggested: 100, 50, 25, 10, 5, 1, 0.5, an 0.2, 0.1, 0.05 and 0.01 million cells/ml. These cel concentrations can be prepared by thoroughly dispersing one-half of a cake of Fleischman's yeast in 100 ml of M/15 KH_2PO_4 and making a cell count with a haemocytometer. A suspension so prepared will have a very high cell count, and suspensions of various cell counts can then be prepared from it by proper dilution with M/15 KH_2PO_4. After each such dilution has been made, it should be thoroughly shaken in a screw-cap bottle with glass beads and a cell count should again be made.

• Exactly 48 or 72 hours after inoculation, the flask is fitted to a distilling bulb with condenser and about 50 ml distilled into a 100-ml graduated cylinder. The distillate should then be made to a volume of 100 ml with distilled water and the approximate alcohol content determined by means of an alcohol hydrometer that is calibrated to read percent by weight of alcohol. Since the hydrometer is usable over a limited range (zero to 4%), it is possible that the alcohol content of the distillate from a flask that received a large inoculum may exceed 4%; if this proves to be the case, dilute the distillate to 200 ml.

Economic Coefficients

The economic coefficient of a fungus is defined as the ratio of the weight (in grams) of the sugar utilized to the weight of fungus mycelium produced, and is generally stated:

$$\text{E.C.} = \frac{\text{sugar utilized}}{\text{mycelium produced}}$$

Economic coefficients have commonly been used as a means of expressing amount of fungus growth and are perhaps among the more accurate ways of determining growth.

- Prepare the following medium: KH_2PO_4, 5 g; Difco yeast extract, 7 g; maltose, 50 g; water, 1000 ml.
- Make a determination of the initial maltose content by means of a Balling hydrometer. This method of determining sugar content yields results that, while only approximate, are accurate enough for the present exercise (for more precise determinations, see Stiles *et al.*, 1926).
- Place five 200-ml portions in 500-ml Erlenmeyer flasks, stopper with cotton and autoclave. Inoculate the five flasks with a loopful of spores of any of the test fungi. Incubate at 30°C.
- As soon as definite mycelial mats have formed on the liquid surfaces in the flasks, remove one flask and filter the mycelial mat on a numbered, weighed filter paper. Determine the approximate sugar content of the filtrate with the hydrometer. Wash the mycelial mat and filter paper several times with distilled water, dry at 95°C and weigh. Calculate the economic coefficient.
- Repeat determinations at two-or three-day intervals until the mycelia, from all of the flasks, have been recovered. For the convenient determination of economic coefficients of unicellular organisms such as bacteria

and yeasts, the organisms may be cultured in numbered, weighed centrifuge tubes of appropriate size. The organisms are then centrifuged out, dried, and weighed in the tube.

Carbohydrate Concentration and Efficiency of Conversion

The efficiency by which a fungus is able to convert substrate carbon to mycelial carbon will determine whether or not a particular fungus is capable of growing in a medium in which the concentration of the carbon-containing compound is very dilute. In general, fungi are very efficient in this respect, as evidenced by the fact that in their natural habitats most fungi grow well. With increase in concentration, however, efficiency of conversion lessens. For most fungi, efficiency varies inversely with the concentration of available carbon compounds in the substrate. This can be simply demonstrated by preparing a basal medium as follows: Difco yeast extract, 1.4 g; KH_2PO_4, 1 g; distilled water, 200 ml.

Four 50-ml portions of the medium are placed in 250-ml Erlenmeyer flasks. Add 0.5 g glucose to one flask, 1.5 g to a second, 2.5 g to a third, and 3.5 g to the fourth. Plug and sterilize by autoclaving. Inoculate all four flasks with nearly equal amounts of inoculum from a slant of *Linderina pennispora* or *Heterocephalum aurantiacum* (any fast-growing, well-sporulating species can be substituted). Inoculum can best be prepared by adding 10 ml of sterile water to a well-sporulated slant, shaking to obtain a good spore suspension, and then pipetting 0.5 ml of the resultant suspension into each of the four flasks. Flasks can then be incubated for about a week without agitation or half that time on a shaking apparatus. At the end of the incubation period, filter the mycelium of each flask on a weighed filter paper, dry at about 60°C, place overnight in a desiccator and weigh. Calculate the economic coefficient in each culture as follows:

$$\text{E.C.} = \frac{\text{weight of sugar in medium}}{\text{dry weight of mycelium}}$$

The economic coefficient value calculated in this manner represents the number of unit weights of sugar required to form one unit weight of dry mycelium under the conditions described. Thus the smaller the value, the more efficiently the fungus is converting substrate carbon to mycelium carbon, and the value should vary directly with sugar concentration. The exercise can be refined by controlling temperature during incubation and by determining if any sugar remains at the time of harvest. If some sugar remains, the amount thereof can be determined and the E.C. calculation can be based upon sugar utilized rather than sugar supplied.

Fungus Enzymes

Determinations of the nature and quantity of fungus enzymes may be made in one of two ways: (1) By culturing the organism on medium containing a particular compound and determining if this compound is utilized, or (2) by preparing extracts of fungus mycelium that contain the various enzymes and testing these extracts for their activity against specific substrates.

Culture Tests The most accurate and most easily conducted tests for enzymes involving growth of the organism on a chemically-defined medium are those in which the substrate compound is broken down to an easily identified substance. The production of colored substances or compounds that affect some indicator in the medium form the basis for tests of this nature.

● Test for Emulsin--Prepare several agar slants with medium of the following composition: $MgSO_4 \cdot 7H_2O$, 0.5 g; K_2HPO_4, 1 g; KCl, 0.5 g; $FeCl_3$, 0.01 g; agar, 20 g; arbutin, 10 g. Make to 1 liter. Inoculate two or three tubes with a selected fungus species. Incubate, and examine at 24, 48, and 72 hours.

● Test for Amidase--Prepare several agar slants with medium of the following composition: $MgSO_4 \cdot 7H_2O$, 0.5 g; K_2HPO_4, 1 g; KCl, 0.5 g; $FeCl_3$, 0.01 g; asparagine, 0.5 g; 2% rosolic acid, 5 ml; agar, 20 g. Make to 1 liter. After this medium is solidified, it should have a slight yellow tint. Inoculate several tubes with the organism used just above and examine at 24, 48, and 72 hours.

Enzyme Extracts Fresh mycelium may be ground or broken in a Waring blender and the extract used immediately for enzyme studies, it may be dried at room temperature and then ground and extracted, or it may be quickly frozen, dried in the frozen state, and then ground and extracted. Of these methods, the last (lyophil method) is probably the best for preservation of enzymes.

Prepare five 250-ml Erlenmeyer flasks each of which contains 100 ml of a standard medium (omit agar). Inoculate all five flasks with one of the following fungi: *Neurospora crassa, Phycomyces blakesleeanus* (+), *P. blakesleeanus* (-), *Rhizopus nigricans* (+), *R. nigricans* (-), *Ascobolus striisporus.* When a good mat has formed in each flask, harvest the mycelia, wash in distilled water, and press between the hands to remove as much water as possible. Cut the pads into 1/4" squares with scissors and place in a lyophil flask with a minimum of distilled water. Shell freeze the flask contents by rotating in a dry ice - methyl cellosolve bath, then lyophilize to complete dryness. The lyophilization process will have to be checked at 30-45 min intervals for an 8-9 hour period. When mycelium is dry, place in a stoppered bottle in the refrigerator.

Blend 3 g of dried mycelium in phosphate buffer solution in a Waring blender, homogenize the suspension, and then centrifuge. Use the clear supernatant in the following tests:

- Phenol oxidase test--Place 5 ml of 0.04% tyrosine in a Klett-Summerson tube, add 5 ml of extract, mix, and measure the optical density, using the Klett-Summerson Photoelectric Colorimeter (other types may be substituted). Remove from the colorimeter, place in a 37°C water bath, and then read again after 15 min. Repeat after 30 min. For a control in this and subsequent tests, substitute 5 ml of extract that has been held in boiling water for 5 min.
- Invertase test--To 5 ml of 1% sucrose solution add 5 ml of extract; cover with toluene (about 1/8" layer) and incubate for 12-24 hours. Test about 1 ml of the water layer with Fehling's solution.

- Amylase test--To 5 ml of 1% soluble starch suspension add one drop of iodine solution and 5 ml of extract. Observe and record the time required for disappearance of the blue color.
- Inulase--Prepare 1% inulin solution for use as the substrate. Place 10 ml in each of two test tubes. To one tube add 5 ml of enzyme solution; to the other add 5 ml of autoclaved enzyme solution. Add 1 ml of toluol to each tube (this serves as a preservative) and incubate at 30°C. At the end of two to four days test with Fehling's solution.
- Esterase--Use a 1% solution of ethyl acetate as the substrate and set up two tubes as above. Incubate for three weeks and titrate the contents of both tubes with N/20 NaOH against phenolphthalein indicator.
- Oxidase--Place 5 ml of enzyme solution in one test tube and 5 ml of boiled enzyme solution in another. Add 0.5 ml of a 10% gum guaiacum solution to each tube, incubate and observe after an hour or two in order to see if a blue color appears.
- Hemicullulase--Place about 1 g of date endosperm slices in each of two test tubes. Add 5 ml of enzyme solution to one tube and 5 ml of autoclaved enzyme solution to the other. Add toluene to each tube, incubate at 30°C for 24 hours, and test the contents of each tube with Fehling's solution.
- Rennet--Place 10 ml of fresh skim milk in each of two test tubes. Add enzyme preparations and toluene as above. Incubate at 30°C for 24 hours, and note whether or not coagulation has occurred.
- Catalase--Add a few drops of hydrogen peroxide to 2 ml of enzyme solution in a test tube; note if bubbles are formed. Repeat, using the autoclaved enzyme solution.

Production of Amylase

For the production of beverage alcohol through the fermentation of starchy grains, it is required by law in the United States that the starch be digested by malt amylase; however, amylase preparations of a high degree of activity can be obtained from certain fungi. Mold-produced amylase is widely used in the Orient and has been used also in Europe. It is produced to a certain extent in this country, and more or less concentrated preparations are sold under the trade name "Taka-diastase."

- Mix 25 g of wheat bran with 25 ml of 0.3N HCl in each of two 500-ml Erlenmeyer flasks, plug and autoclave for 30 min.
- Inoculate each flask heavily with spores of *Aspergillus oryzae*; stir so that the spores are spread throughout the bran. Incubate the flasks (lying on their sides) at 30°C until sporulation has just started, then pour the moldy bran onto heavy paper or a glass plate and allow it to dry at room temperature. When the moldy bran is dry, grind in a Wiley mill and store in a screw-cap bottle.
- For the determination of amylase activity, prepare a 4% soluble starch "solution" and from this prepare (by dilution) 2%, 1%, 0.5%, and 0.25% soluble starch solution.
- Weigh 1 g samples of moldy bran into each of five 1" x 8" test tubes. Add a 10-ml portion of one of the different starch solutions to each tube of the series. This should be performed as rapidly as possible so that the starting time will be approximately the same for all tubes. Add 1 ml of starch indicator to each tube and incubate at 30°C. Note the time at which the moldy bran and starch solutions are mixed and observe the tubes at intervals in order to note the time of blue color disappearance in the various tubes.
- If ground moldy bran is placed in a funnel on coarse filter paper and water allowed to percolate through it, the percolation water will contain amylase that can be precipitated by the addition of alcohol. The precipitate can be further purified by repeatedly dissolving in water and precipitating with alcohol. If time permits, a small amount of the enzyme concentrate should be so prepared and tested as above.

Production of Acetaldehyde

- Prepare 100 ml of medium containing 0.5 g KH_2PO_4, 0.7 g Difco yeast extract, 5 g glucose. Place a 10-ml portion in a 50-ml Erlenmeyer flask and a 90-ml portion in a 250-ml Erlenmeyer flask; stopper with cotton and autoclave.

- Inoculate the 10-ml portion from a slant culture of *Hansenula anomala*; incubate at 30°C for 24 hours.
- Inoculate the 90-ml portion with the 24-hour culture in a small flask and incubate for 6 days at 30°C.
- Pipette a 50-ml sample from the six-day-old culture and distill about 25 ml into an ice-cooled 50-ml Erlenmeyer flask.
- Place 5-ml portions of the distillate into each of two clean test tubes.
- Dissolve about 0.5 g of sodium bisulphite in the distillate in one tube; then add 1 ml of a 2,4-dinitrophenylhydrazine hydrochloride solution to each tube. Observe.

Synthesis of Ethyl Acetate

- Prepare 200 ml of the following medium: KH_2PO_4, 0.5 g; Difco yeast extract, 0.7 g; glucose, 5 g; water, 100 ml. Place 90-ml portions of the above in each of two 250-ml Erlenmeyer flasks and 10-ml portions in each of two 50-ml Erlenmeyer flasks. Plug and autoclave.
- Inoculate both 10-ml portions from a slant of *Hansenula anomala*; incubate at 30°C for 24 hours and then inoculate each 90-ml portion with a 10-ml portion. Incubate at 30°C
- After five days' incubation remove one flask and pipette out a 50-ml sample. Distill about 25 ml of this sample into a 500-ml Erlenmeyer flask, add a few drops of phenolphthalein indicator, and neutralize by adding 0.1N NaOH one drop at a time (color should be just pink).
- Add 25 ml of 0.1N NaOH to the neutralized distillate and reflux for one hour. Cool and titrate with 0.1N HCl. Total ethyl acetate produced (milligrams) = (25 minus ml of HCl) x 8.8 x 2.
- After 10 days' incubation, remove the second flask and analyze for ester as above.

Biosynthetic Deficiencies

Fungi vary greatly in their capacity to synthesize various of the B-complex vitamins and essential amino acids.

Some species are able to synthesize all of the vitamins and amino acids, while others may be "deficient" for one or more such compounds. Fungi known to be deficient for various of the B-vitamins or amino acids have been used successfully as test organisms in biological assays for such materials. Of particular interest in this respect are certain mutant strains of *Neurospora crassa*. The techniques employed here are in general applicable to work involving strains that are deficient for other amino acids or vitamins. (For this exercise use *N. crassa* Strain 33756-4637A, a leucineless mutant.)

All glassware used must be *scrupulously* cleaned. Use only double-distilled water throughout this exercise. Since vitamins and amino acids are effective in very minute quantities, extreme care must be exercised in the preparation of medium that is to be used in demonstrating a deficiency. Common sources of minute amounts of such materials are the other components of the medium; these, therefore, should be purified before use. The complete medium is compounded as follows: NH_4NO_3, 1g; KH_2PO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; sucrose, 15 g; biotin, 0.2 gamma; leucine, 2.0 mg. Make to 1 liter. Dissolve the first four ingredients in about 900 ml of distilled water, add Norit A at a level of 1 g/50 ml solution, boil for 10 min and filter. Cool and make to 1000 ml with distilled water. Add biotin and leucine in proper amounts to one portion, then pipette 10-ml quantities into each of three clean French square bottles. Plug, autoclave, and inoculate. To another portion add only biotin; prepare three French square bottles and inoculate.

Prepare another set of three bottles with medium containing only the first four ingredients. *Do not* treat with Norit and do not add biotin or leucine.

Incubate for 4 days at 30°C and observe.

Decomposition of Wood

- Number and weigh (to the nearest 0.1 g) five air-dry wood blocks (1/2" x 1/2" x 3") of the same type of wood. Record each block's weight.
- Place weighed blocks in distilled water and autoclave for 30 min at 15 lb pressure in order to thoroughly saturate with water.

- Remove blocks from water and place each in a 1" x 8" test tube. Add 10 ml of mineral salt solution (see below) containing a small amount of corn steep to each tube. Plug and autoclave for 30 minutes.
- Inoculate all tubes with one of the common wood-destroying fungi. Inoculum should consist of a small block of agar covered with mycelium; the inoculum should be placed on the wood block at the liquid-air juncture.
- Incubate for six weeks, remove the blocks, and carefully wash to remove any surface mycelium. Dry and weigh. Calculate percentage weight loss. Compare with different fungi and different types of wood.

An alternative method for examining decomposition of wood is as follows:

- Prepare 3% malt agar (25-30 ml per Petri dish).
- Inoculate with fungus and allow colony to become established (about one week).
- Sterilize wood blocks (1/2" x 1/2" x 1") in water at 15 lb for 20 min.
- Place cooled blocks in culture dishes on the mycelium.

Cellulose Degradation

- Cut 1-1/2" x 5-1/2" strips of 12.29-oz untreated gray duck with warp threads in the long dimension. Ravel from both sides of the long dimension until each strip is exactly 1" wide.
- Wet the ravelled strips thoroughly with distilled water and place each in a 1" x 8" test tube. Add 5 ml of mineral salt solution to each tube, stopper with cotton and autoclave.

Note--A mineral salt solution is made as follows:

KH_2PO_4	5.0 g
$MgSO_4 \cdot 7H_2O$	0.5 g
NH_4NO_3	0.6 g
*Trace element solution ...	1.0 ml
**$FeCl_3$ solution	1.0 ml
Make to 1000 ml	

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*H_3BO_3, 0.114 g; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.484 g; $CuSO_4 \cdot 5H_2O$, 0.780 g; $MnCl_2 \cdot 4H_2O$, 0.144 g; $ZnSO_4 \cdot 7H_2O$, 16.720 g; make to 1000 ml.

**1.92 g $FeCl_3$ made to 1000 ml.

- Inoculate each strip by making a single vertical streak 1" long at about the middle of the strip. For inoculum use spores of *Myrothecium verrucaria* or *Chaetomium globosum*.
- Remove a portion of strips at the end of 4, 8, 12, 16, and 20 days. Wash to remove as much fungus mycelium as possible, dry, and determine tensile strength by breaking in a Scott Testing Machine. Calculate percentage loss of tensile strength.

Production of Penicillin

Submerged Culture This method is generally employed on a laboratory scale, the mold growing throughout the liquid in pellet form. Aeration and agitation of the medium are necessary for good results. Other media may be substituted for the one listed below. A strain of the *Penicillium notatum-chrysogenum* group should be used.

- Prepare the medium (Moyer and Coghill, 1946) as follows:

Corn-steep liquor	40.0 g
Lactose monohydrate	37.5 g
Glucose monohydrate	3.0 g
$NaNO_3$	3.0 g
$MgSO_4 \cdot 7H_2O$	0.25 g
KH_2PO_4	0.50 g
$ZnSO_4 \cdot 7H_2O$	0.044 g
$MgSO_4 \cdot 4H_2O$	0.020 g
Distilled water to make	1000.0 ml

- Adjust pH to 5.6. Dispense the medium into 250-ml Erlenmeyer flasks at the rate of 100 ml per flask. Sterilize.
- After the flasks are cool, add 1-1.5 g of sterile calcium carbonate to the flasks. The concentration is generally 1% of the solution. Check the pH.
- Inoculate the medium with suspensions of spores. Germinated spores in the form of pellets may be used to speed up penicillin production.
- Place the flasks in a shaking machine at 200 revolutions or cycles per minute, at a temperature of about 24°C.
- After six to seven days, remove the flasks, and recover the mycelium by filtration.

Recovery Recovery of penicillin is based on solubilities, formation of salts and crystallizing out by drying.

- Mix the filtrate with a solvent such as amyl acetate, cool to low temperature, and agitate vigorously while adjusting the pH (30:1 partition coefficient of amyl acetate or butyl acetate).
- Acidify broth to pH 2-2.5 with acid such as phosphoric acid. This transfers the penicillin to the solvent as an acid. This step must be completed rapidly to avoid breakdown of the unstable penicillin at this pH.
- Centrifuge the solution (with a surface-active agent to prevent sludge formation due to protein precipitates).
- Agitate the solvent and water while alkali (or buffer) is added to bring the pH to 7 or 7.5. The penicillin now passes into the aqueous phase as the sodium salt. Separate by gravity or centrifuge from solvent.
- Take up penicillin with another solvent in a manner similar to first extraction.
- Extract in small amount of water with NaOH (or sodium bicarbonate) if the sodium salt is desired (if calcium salt is preferred, use calcium in place of sodium). Centrifuge.
- The penicillin may be vacuum dried, or it may be evaporated in the frozen state.
- Check yields by assay method on agar plates.

See also Prescott and Dunn, 1959.

Preparation of Tempeh

While soybeans have a high protein content and thus are nutritionally valuable, they are difficult to digest and contain a pancreatic hypertrophy factor. Hence soybeans as such have been little used for human food. However, in the Orient, soybeans that have been processed with fungi of various types have been eaten for centuries. One such type of food is known as "tempeh." During the production of tempeh, the soybeans are rendered more digestible and more tasty, and the pancreatic hypertrophy factor is removed or destroyed. Tempeh can be easily made in the laboratory as follows:

- Obtain a volume of soybeans equal to two full Petri dishes (Cutler variety works especially well).

- Crack soybeans in half and soak in tap water for a minimum of eight hours. Use more than three times the volume of the beans. Rinse away separated hulls.
- Cook in clean water for 30 min (after water comes to boil). Pour off water. Place beans on clean paper towels to drain and to cool to room temperature.
- When cool, put beans back in cooking vessel. Inoculate beans with 5-ml suspension of spores of *Rhizopus oligosporus* (NRRL 2710). In addition to the strain of choice, *R. oligosporus*, three other species, *R. stolonifer*, *R. oryzae*, and *R. arrhizus*, have been used to make tempeh.
- Place inoculated beans in Petri dish and replace cover. Dish should be filled but not packed tightly. Incubate at 30°C for about 20 hours. Start checking after 15 hours. If spores become visible before 20 hours, remove from incubator, wrap in aluminum foil, and refrigerate. Tempeh can be easily handled at this stage since it is a solid cake held together with white mycelium. During the incubation period, most of the soybean is solubilized by the fungus and the hyphae bind the particles together in a single mass. The final product is similar to cheese, although not as uniform in texture.
- Cut in thin slices, salt, and cook in deep fat (peanut oil is especially good) until light brown. Eat as soon as it has cooled enough.

In place of Petri dishes, perforated plastic bags may be filled with inoculated soybeans and incubated. The perforated plastic bags with the inoculated soybeans may be refrigerated or deep frozen, then removed later for 36-38 hours for the frozen beans or 21-22 hours for the refrigerated beans before fermentation.

If time permits, some other legume, such as dried peas, lima beans, etc., may be processed in similar manner.

See also Hesseltine and Wang, 1967.

BIOLOGY OF MARINE FUNGI

It is important to emphasize that marine fungi are not uniquely adapted structurally to seawater and that their general morphology parallels that of fungi from other habitats. Detailed information regarding many groups that are to be found in the marine environment is to be found in Part II of this Guidebook.

Members of the Labyrinthulales are very easily obtained from leaves of eel grass (*Zostera*), still relatively abundant on all coasts. Labyrinthulas can be most readily secured for class use by culturing (see p. 38-40).

The two principal sources of marine Phycomycetes are algae and sediments. To find representatives on algae is time consuming, and the yield often does not justify the effort. Obtaining algae at the "best season" may be difficult, for information as to when to collect likely algae is generally lacking. Mucoraceous species from sediments are common terrestrial forms, for the most part, and therefore more easily obtainable from soil. Culturing methods are known, but yield depends on rather careful and precise techniques and use of complex media.

Thraustochytrium and *Schizochytrium*, two monocentric members of the Saprolegniales, are easily obtained for laboratory examination. Pieces of marine algae about 2 cm^2 are placed in 100 ml of seawater, and sterile pollen of gymnosperms such as *Pinus* or *Cedrus* is added. In about three days the thalli of these two organisms will be visible on the pollen when examined under the dissecting microscope. For detailed examination, mount the pollen in seawater, add a cover slip, and examine with the compound microscope. Pure cultures of *Thraustochytrium* and *Schizochytrium* are readily established by streaking pollen with the fungi on the antibiotic-containing "isolation medium" used by Fuller *et al.* (1964). An alternate method for obtaining these two organisms and other marine fungi is to plate pieces of algae directly on the medium. Plates with algae on them will also show *Labyrinthula* sp. in about a week.

The Trichomycetes, a rather special group of mostly marine fungi, are found in association with invertebrates and are discussed in detail in Chapter 9.

The most easily collected and maintained fungi from salt water are the Ascomycetes and Fungi Imperfecti. Only two species of Basidiomycetes are known; neither is common or easily recognized. Yeasts are abundant in coastal waters. Fungi in marine invertebrates are highly seasonal, occurring, generally, during the summer breeding period of the animals. Maintaining collections is difficult, and preserved specimens are unsuitable for general student use. In summary, the subject and treatment of marine fungi can best be built around a consideration of Ascomycetes and Fungi Imperfecti, with one or two additional special groups included to illustrate diversity.

Collecting

Principal sources are: Wood, either as drift or as submerged, 4" x 3/4" panels; dead culms of grasses such as *Spartina*; and various marine algae. In coastal areas, such as the Southeast, where there is little driftwood, wooden blocks or panels can be submerged and tied to pilings. For routine collecting the wood need not be sterilized. Basswood, any common coniferous wood, or any common building or furniture wood can be used. Drill a 1/4" hole in the center of each panel. Push a 3/16" x 2" iron stove bolt through the hole of one panel and into the hole of a second panel, tie (by a simple knot) the two panels to a 1/4" polyethylene line and tighten the panels to the line by screwing down the nut. This provides a "sandwich" of two panels, separated by the width of the line. Fasten one end of the line to a concrete construction block and the other end to a piling or pier runner. Several panel pairs can be fastened in series to the line; the concrete block will prevent the submerged line of panels from drifting.

Submergence time varies, depending roughly on season. One or two months is sufficient in temperate waters in the summer; three to four months should be allowed in colder waters. In some coastal areas, fouling is high but is of no concern. Fouling organisms can be scraped with a putty knife from submerged wood, leaving the fungi intact.

Driftwood in colder coastal waters is a particularly fruitful source. Pieces can be picked up from coastal embayments, or pieces wedged in rocks by tidal action may be used. For ease of handling, large logs may be chipped with a hand axe or knife and the chips brought in. Driftwood with bark intact is generally unsatisfactory, and bark itself seldom harbors marine fungi. Wood buried in sand or mud is not suitable, although wood lying on the bottom (not covered by silt) is an excellent source.

The most common representatives of Ascomycetes are species of *Lulworthia*, *Ceriosporopsis*, and *Halosphaeria*; *Helicoma* (= *Zalerion*), *Cirrenalia*, and *Dictyosporium* species constitute the major deuteromycetous flora. Dead culms (not leaves) of phanerogams commonly harbor *Lulworthia*, *Leptosphaeria*, *Phoma*, and *Sphaerulina*. Fructifications of these are easily seen with the unaided eye.

There is no simple rule-of-thumb method for locating these fungi on wood. The "hit or miss" method is best. Students can be given bits of submerged wood and should scan them, mounting and examining likely bits of material. They soon learn to recognize fungi as distinct from various bits of detritus clinging to the wood surface.

In Europe, seaweeds--particularly members of the Fucales--are heavily colonized by a whole series of Pyrenomycetes and Coelomycetes, which are easily demonstrated to students.

Laboratory Study

A strong light source and a dissecting microscope are essential. Fructifications can be located by scanning the wood or culm at medium or high magnification. Ascocarps are easily recognized; conidia appear as minute, black, "pepper-like" aggregates on the substrate surface. A flattened, spear-point needle is recommended for use in digging the fructifications from the substratum. Ascocarps or conidia can be mounted directly in drops of seawater on slides. For best observation of spore appendages, high dry magnification is recommended, with the field illumination reduced. Such appendages are better observed in unstained than in stained mounts. Most Ascomycetes from seawater have deliquescent asci, and asci are therefore not likely to be found in most preparations. Ascomycetes on culms, however, generally have persistent asci.

Shipment and Maintenance

Wood panels or driftwood may be shipped air mail from source to laboratory, with minimum difficulty. Substratum pieces should be separately wrapped in aluminum foil or sheet plastic; the wood is usually sufficiently wet to permit shipment without additional water. Substrates are best maintained for class use in running seawater, but large battery jars of seawater may be used. Only a few pieces of wood or culm should be put in the jars, and the water should be aerated. If seawater is not obtainable, tap water containing 3% NaCl may be substituted. Panels and culms may, of course, be dried or preserved in 5% formalin in seawater, but there is always some loss of spore detail in preserved material, and it is often difficult to locate fructifications on dry wood. This can be overcome to some extent by locating clusters of fruiting bodies on freshly harvested wood, marking these with a pin, drying it, and then rewetting in the laboratory. A 3% NaCl solution is recommended for rewetting.

Culture

Only a few Ascomycetes from marine habitats lend themselves easily to culture. Fewer still are known to produce ascocarps in culture; *Ceriosporopsis halima* and species of *Lulworthia* fruit most readily in the test tube, although others will produce fruiting bodies after incubation for three to six months. Some species that produce ascospores with gelatinous appendages form abnormal or reduced processes in culture.

The most satisfactory culture medium involves wood embedded in agar as follows: Aged seawater, 1 liter; yeast extract, 0.3 g; KNO_3, 0.2 g; K_2HPO_4, 0.2 g; disodium succinate, 0.02 g; biotin, 0.4 μg; thiamin HCl, 8 μg; tris buffer, 1 g; trace elements, 1 ml; agar, 18 g. Trace element solution contains, per milliliter,

Na_2EDTA, 30 mg; $Na_2MoO_4 \cdot 2H_2O$, 0.01 mg; chlorides: Fe^{+3}, 1 mg; Zn^{+2}, 0.3 mg; Mn^{+2}, 0.5 mg; Cu^{+2}, 0.02 mg. Heat seawater to dissolve agar, add nutrients, and stir to dissolve. Dispense 10-ml amounts into 20 x 150 mm screw-capped culture tubes, add one or two birch applicator sticks to each tube, autoclave and slant. The applicator sticks may be obtained from any drugstore.

To secure inoculum, flame-sterilize a fine-pointed needle and with it puncture an ascocarp on wood. Pick up a mass of extruded spores with another flame-sterilized, cooled needle, and transfer the spore mass to the base of the wooden sticks near the agar surface. Within three to five weeks, bacteria-free ascocarps will develop on the wood substrate. Often, larger fructifications on driftwood or wood panels will exude spores naturally, in a cirrhus, if allowed to become partially dried. This mass is an excellent source of bacteria-free spores for inoculum.

The medium may also be used without agar. In this case, prepare and dispense as indicated above, add the birch sticks, and, after autoclaving and inoculation, slant the tubes. When mycelial growth is well established, the liquid may be poured off; some species fruit more readily under these conditions. Incubation temperature for all cultures should be between 20° and 25°C. Aged seawater is easily prepared by storing raw seawater in carboys, in the dark, for at least four weeks.

The foregoing technique will not give unisporal cultures and in some instances will not yield bacteria-free cultures. The fructifications that develop will, however, be characteristic of those developing on submerged wood and are preferable for class use. This medium will support Fungi Imperfecti as well as Ascomycetes. Most Ascomycetes develop very slowly to the perfect stage, hence maximum suitability is usually not reached before one month's incubation.

Ascomycetes can be isolated (by spore-mass transfer) on an agar medium containing, per liter of aged seawater, 0.1 g yeast extract, 1 g glucose, and 18 g agar. Pour plates, and inoculate in the center. Large colonies will develop and will be free of bacteria at the outer edge. Very few species will produce ascocarps on this medium, but mycelium from these colonies can be transferred to the agar-birch medium. In most instances, subsequent growth will result in ascocarps.

Identification

For rapid, visual identification, the best source of literature is Kohlmeyer (1961). Pertinent pages of drawings and the corresponding species list can be duplicated for class use. Johnson and Sparrow (1961) is a compilation of information on all marine fungi up to 1961. This source, too, includes material for lecture or discussion.

Special Groups

The controversial lime-loving "cladochytriaceae" described by Porter and Zebrowski (1937) may be of interest. These "fungi" are easily obtained and present no problems in preservation or observation. Coarse sand containing very small bits of broken shell can be collected from any beach, either on the surface or from depths up to 6" below the surface. Pick out small bits of shell (not sand), mount them on a glass slide in a small drop of Hoyer's solution or Canada balsam. When the mounting medium is dry, the preparation is ready for observation. Flat pieces of shell are infinitely better than irregular, roughened ones. For observation, place the slide on the stage, and focus with the low-power objective. Light intensity must be adjusted. For opaque pieces, use high intensity; for translucent shells, use a diffuse field of light. The "fungi" can be seen on the surface or immediately below. Dissolving the calcareous material is unnecessary and often destroys the preparation.

SAMPLING AIRBORNE FUNGI

Isolation by Culture

The simplest way to obtain fungi from the air is to prepare nutrient plates and expose at intervals up to

10 min. Incubation at 21°C or at room temperature will result in sporulating colonies in a week or less. While not quantitative, this simple method will give good comparative data. Exposures can be made outdoors and indoors. Since fungus spores are seasonal, the greatest numbers occurring during the growing season, samples obtained outdoors during July and August will be most striking.

Any standard medium such as potato glucose agar, cornmeal agar, malt agar, or Czapek's may be used. When these media are used, bacteria will also be present and, at times, bacterial species that spread rapidly are troublesome. Fast-growing fungus species may also overrun the colonies of other types.

Antibiotics can be used in the media to eliminate the bacteria, and rose bengal will reduce fungus growth and keep the colonies small. An excellent medium is rose bengal streptomycin (M-42) as used by Farrow (1954); most fungi grow on it and sporulation is apparently not affected. Nonsporulating colonies can be transferred to other media. Media containing rose bengal should not be exposed to full sunlight. Exposure causes a photodynamic process such that it will not support fungus growth. Ordinary laboratory lighting is not harmful.

The plates may be held vertically or laid flat on a table or windowsill. Controls should be run to check sterility and technique by allowing the plates to remain two days at room temperature and discarding contaminated plates. Controls on the exposure technique are run by removing the lid and immediately replacing it. Numbers in the control technique plates should be subtracted from the total in the exposed plates.

A summary of fungi obtained on rose bengal streptomycin agar plates exposed outdoors daily for two years is given by Kramer *et al.* (1959), who identified 89 genera. Although a slit sampler was used, the fungi obtained by manual methods would be similar. The commonest fungi were *Cladosporium*, 45%; nonsporulating forms (chiefly haploid Basidiomycetes and some Ascomycetes), 18%; *Alternaria*, 13%; yeasts, 9%; *Penicillium*, 6%; *Aspergillus*, 5%; *Fusarium*, 0.8%; *Pullularia*, 0.7%; *Geotrichum*, 0.4%; *Sporobolomyces*, 0.4%; *Helminthosporium*, 0.3%; *Coniothyrium*, 0.3%; *Phoma*, 0.2%; *Sporotrichum*, 0.2%; and *Rhizopus*, 0.1%.

Cladosporium was the commonest fungus: *C. cladosporioides*, the commonest species, accounted for over 50% of the colonies of this genus; *C. herbarum*, one third; and *C. macrocarpum* and *C. sphaerospermum*, the remainder. *Alternaria tenuis* was the common species of this genus, but on rare occasions *A. cucumerina* and *A. consortiale* were found. Forty-one species of *Penicillium* were found, with *P. oxalicum* constituting 56% of the colonies. There were 23 species of *Aspergillus*, with *A. amstelodami* and *A. niger* contributing 32% and 23%.

Fungi Imperfecti were the most numerous, with 63 genera. There were 7 genera of Phycomycetes, 13 of Ascomycetes, and 6 of Basidiomycetes. The last are very difficult to identify. Many of the nonsporulating colonies are probably basidial, especially if on plates that are exposed at night or early morning, when basidiospores are abundant.

Isolating Fungus Spores

A common method of isolating spores is to expose an adhesive-coated glass slide to the wind for a period of hours. In some studies the slides have been exposed horizontally; in others, vertically or at an angle. Clothespins are satisfactory holders. One of the better methods is to mount the slide in a holder at a 45° angle at the end of a rod with a windvane at the other end. A nail at midpoint set in an upright pipe will allow the rod to rotate and thus keep the slide facing the wind. Exposure for 24 hours will usually give a good sample. Vaseline and silicone grease have been used; the latter has the advantage that it will not wash off and will remain sticky at all temperatures.

The number of fungus spores that can be identified is limited compared with the number obtained by culture. The fungus spores found on adhesive-coated slides exposed over a two-year period have been summarized by Kramer *et al.* (1959). The commonest forms were *Cladosporium*, 41%; basidiospores, 24%; yeasts, 7%; smuts, 6%; two-celled hyaline spores, 4%; *Alternaria*, 3%; *Fusarium*, 3%; one-celled hyaline spores, 1%; *Cercospora*, 1%; fusiform ascospores, 0.9%; *Septoria*, 0.4%; two-celled brown forms, 0.3%; *Leptosphaeria*, 0.2%; *Helminthosporium*, 0.2%; *Pleospora*, 0.2%; rusts, 0.2%.

An invaluable aid to fungus spore identification is found in Gregory's recent book (1962). It has four plates of spores, drawn to scale and colored to show the main features. Spores of *Curvularia, Ganoderma, Polythrincium, Stemphylium, Sporormia* and *Trichothecium* are less common but are easily recognized. Many others can be identified by using Gregory's plates.

Hyphal fragments are common, especially of the dematiaceous types. Many of the fragments are probably broken conidiophores.

Quantitative Sampling

Quantitative data require special sampling apparatus, calibration of the equipment, counting, identifying, and determining numbers of spores or colonies on a volumetric basis. Many ingenious and efficient air samplers have been developed, each with advantages and disadvantages. Two reviews of quantitative sampling methods are available, Gregory (1962) and Wolf *et al.* (1964).

Many samplers will expose sterile plates to the air (Gregory, 1962; Wolf *et al.*, 1964). A convenient and satisfactory sampler is the G.E. Electrostatic Bacterial Air Sampler (Luckiesh *et.al.*, 1946). It is a compact, self-contained portable box that accepts two culture plates. Air is drawn over the plates and the spores precipitated onto the surface. While it can be used outdoors, it is especially useful indoors to determine spore loads in different rooms.

One of the more efficient new samplers is the Andersen Sampler (Andersen, 1958). Six agar plates are placed in a pyramid stack separated by perforated disks that have a series of progressively smaller holes. Air is drawn through the holes at increasingly greater speeds, efficiently separating the spores on the basis of size. Larger spores like *Alternaria* are retained on the first plates, while plates #5 and #6 retain the smaller spores, *Penicillium* and *Streptomyces*. Although efficient, it must be loaded and unloaded in a near-sterile room and is not so convenient as the G.E. instrument. Exposures of 2 min outdoors near midday have been found satisfactory.

A recent development is the continuous type sampler that will sample through a 24-hour period or longer. The Hirst Spore Trap is one of the best known (Hirst, 1952; Gregory, 1962). Sampling is continuous and the slide moves slowly past the orifice for 24 hours. It is operated on household electric current, which limits its usefulness in the field.

A new continuous sampler has been developed that is similar to Hirst's but differs in that the slide does not move continuously. Spores are deposited in bands, one band per hour, which facilitates reading. Sampling can be intermittent or continuous. A 10" sample has been found generally satisfactory, with three periods of 3-1/3 min during an hour. The sampler is known as the Kramer-Collins Spore Sampler, which can be operated on household electric current or from a 12-volt battery.

Spores caught on slides may be observed under low or high power, with or without a cover slip. Lactophenol clears and expands the spores; if a little cotton blue is added, hyaline spores and germ tubes will be stained. The preparation is not permanent, however, and bubbles often develop if the mount is left overnight. With a little experience the spores of most fungi can be identified dry without a cover slip and the slides can be stored indefinitely.

Laboratory Study

Spore Content of Buildings Exposing plates 2-5 min in different rooms will give interesting results. Plates can be taken to other buildings on university campuses, especially to dining halls, kitchens, or field houses. Samples in a greenhouse will yield large numbers of colonies. Samples from animal rooms make good comparisons. *Penicillium* is the commonest fungus indoors, just as *Cladosporium* is the most abundant outdoors.

Selective Action of Media Several different media can be exposed simultaneously. Media containing antibiotics are very useful (Farrow, 1954). Unusual media can be tried. In general, bacteriological media do not support good fungus growth but make excellent comparisons.

Isolation of Thermophilic Fungi Incubation of plates at 50°C will allow thermophiles to grow. Some will grow at 60°C. Thermophilic fungi are abundant in moldy hay and in compost piles and their spores are present in the air.

Diurnal Periodicity Exposures made throughout the day and night will show a wide variation in numbers and kinds of fungus spores. Gregory (1962) recently summarized the evidence on diurnal periodicity. He recognizes five patterns: (a) Diurnal, with peaks at 6:00 am and 6:00 pm; (b) nocturnal; (c) forenoon; (d) afternoon; (e) evening. Most Fungi Imperfecti appear to belong in (c) and (d), Basidiomycetes and Ascomycetes in (b). Most studies on periodicity have been made by using continuous spore samplers, but they can be done also with nutrient plates. No machines are known that will make continuous samples using sterile nutrient plates, so exposures must be done manually.

Meteorological Changes Gentle rains will gradually remove spores from the air but are followed immediately by release of ascospores. Samples taken before, during, and after a rain, with either adhesive-coated slides or nutrient plates, will show striking differences in numbers and kinds.

Spore Viability This variable can be tested easily after the spores have been caught on the adhesive-coated slide. A slit sampler is useful because it deposits the spores in a band where they may be easily located. A simple technique described by Pathak and Pady (1965) uses silicone-coated slides. After exposure, the spore deposit is atomized with sterile water and placed in a moist chamber for 24-48 hours. Slides are air dried and may be stored indefinitely. Spore germination is good and the germinated spores are readily located by their germ tubes. Viability can be determined by comparing numbers of germinated and nongerminated spores. Since *Cladosporium* is the commonest spore type and germinates readily, it is a good form to use. Germinating rust spores and hyphal fragments make an interesting demonstration.

Secondary spores are occasionally formed on a germination slide. The germ tube becomes a conidiophore and produces a cluster of spores. *Cladosporium* does this frequently and the branching treelike conidiophore with olive brown spores in chains is striking.

Sampling in Winter Silicone-coated slides can be exposed for long periods during cold weather, as silicone remains adhesive at very low temperatures. Obtaining cultures from air at near zero F, or lower, poses problems, since agar freezes at this temperature. One method that has been used successfully is as follows: Coat the inside of a Petri dish with a thin layer of silicone grease, add a lid, and sterilize in an oven. The sterile plates may now be exposed for as long as desired. When the plates are brought into the laboratory, add warm agar. The fungi germinate and grow through the overlying agar to produce colonies on the surface.

See also Dimmick and Akers, 1969.

HYPOGEOUS MACROFUNGI

Although laymen have generally heard only of the truffles (order Tuberales), hypogeous fungi are very diverse. They are to be found in many families and genera of the Basidiomycetes, Ascomycetes, and Phycomycetes (Table 6), and the general principles of finding, describing, preserving, and culturing them apply to most species. Most are probably mycorrhizal and accordingly necessary for the wellbeing of their vascular hosts. Many are prized for their fine eating qualities and most seem to be avidly sought as food by animals.

Though abundant in much of North America, hypogeous fungi are seldom encountered by those collectors who seek specimens only on or above the ground surface. Once one learns the tricks of finding these fungi, however, he need rarely return empty-handed from a search. Moreover, the likelihood of turning up new taxa offers attractive and exciting incentive, since little of the world has been well explored for hypogeous fungi.

TABLE 6 Representative Taxa Having Hypogeous Members

CLASS	Order Family	Genus	
ASCOMYCETES	Eurotiales		
	Elaphomycetaceae	*Elaphomyces*	
	Tuberales		
	Geneaceae	*Genea*	
		Genabea	
	Terfeziaceae	*Delastria*	*Tirmania*
		Picoa	*Terfezia*
	Tuberaceae	*Balsamia*	*Hydnotrya*
		Barssia	*Pachyphloeus*
		Choiromyces	*Stephensia*
		Geopora	*Tuber*
		Hydnocystis	
BASIDIOMYCETES	Hydnangiales		
	Hydnangiaceae	*Arcangeliella*	*Macowanites*
		Cystangium	*Martellia*
		Elasmomyces	*Octavianina*
		Gymnomyces	*Zelleromyces*
		Hydnangium	
	Hymenogastrales		
	Hymenogastraceae	*Chamonixia*	*Protogautieria*
		Gautieria	*Rhizopogon*
		Hymenogaster	*Richoniella*
		Mycolevis	*Truncocolumella*
	Hysterangiaceae	*Hysterangium*	
		Phallogaster	
		Rhopalogaster	
	Melanogastraceae	*Alpova*	*Leucogaster*
		Cremeogaster	*Melanogaster*
	Secotiaceae	*Gastroboletus*	
		Thaxterogaster	
	Lycoperdales		
	Lycoperdaceae	*Abstoma*	
		Radiigera	
	Sclerodermataceae	*Scleroderma*	
	Sedeculaceae	*Sedecula*	
PHYCOMYCETES	Mucorales		
	Endogonaceae	*Endogone*	

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Not surprisingly, the known distribution of hypogeous fungi fairly well coincides with the working areas of mycologists who have specialized in studying them. Thus, in North America by far more species have been discovered in California, Oregon, Washington, and Idaho than in the entire rest of the continent. Most of the world's knowledge about hypogeous fungi is derived from exploration of less than one-fifth of the land surface lying between 40° and 60° N latitude. It seems safe to assume that hypogeous fungi are abundant in both number and species throughout forested parts of the North Temperate Zone. Limited collections from the southern hemisphere suggest that the same may hold true for the South Temperate Zone. So little collecting has been done either in the arctic or in the tropics that there is no basis for speculation.

Hypogeous fungi are typically found near mycorrhizal roots in forests, groves, and shrub areas. Some species appear to be relatively nonselective in terms of mycorrhizal host species; for example, *Melanogaster ambiguus* and *Tuber puberulum* associate with trees in several genera and families. Other hypogeous fungi seem to require hosts from a restricted number of genera. Examples of narrow host specificity include *Alpova cinnamomeus* with *Alnus* spp. and *Tuber melanosporum* with *Quercus* spp.

In habitats that remain moist throughout the summer, hypogeous fungi often grow in the litter layer or, occasionally, so close to the surface that they emerge at maturity, particularly along stream banks or other places where the water table remains high. Some vernal species develop on the humus surface under snow banks, to be revealed as the banks recede in spring. More typically, however, hypogeous fungi reach maximum development in species and numbers near the interface between humus and mineral soil--it is here that mycorrhizae develop most profusely. A few, such as certain species of the genera *Tuber* and *Elaphomyces*, characteristically fruit more deeply in the soil; some, indeed, have been found at depths of several feet.

Certain microhabitats appear to be particularly congenial for some species. *Hydnotrya variiformis* fruits profusely in half-buried, very rotten conifer logs. *Hymenogaster alnicola*, an apparent mycorrhizal associate of *Alnus* spp., can occur in soils so boggy that one would hardly expect to find any such organism.

Every kind of seasonal occurrence is represented among the hypogeous fungi. In temperate regions there is usually a flush of fruiting in spring and autumn, somewhat less so in summer, depending on weather and habitat (in the higher mountains most abundant fruiting typically occurs in summer). Many species normally fruit in one particular season, while others appear sporadically or more or less continuously throughout the growing season. The Basidiomycetes tend to be relatively ephemeral; the primordium forms, expands, and the sporocarp matures and decays in a relatively short time. Many Ascomycetes, by contrast, take several months to mature and then decay only slowly. Such fungi can be found throughout the growing season but often cannot be identified for lack of spores until late summer. *Elaphomyces* spp. are particularly durable and may even overwinter to mature in the season following initial expansion of the primordium.

Collecting

The most important tool for unearthing hypogeous fungi is a fork or rake. Preferences vary, but in general a four-tined garden cultivator with the handle cut to a comfortable length (about 30") is most efficient. Hand cultivators are also satisfactory but require more stooping and cover less area than the larger type. Once a likely spot is chosen for examination, the litter is gently raked aside and the exposed humus inspected for specimens. It, too, is than raked away down to mineral soil. During and after each stroke of the fork, the material being worked should be carefully watched. Having reached mineral soil, the collector will often find it worthwhile to rake several inches deeper, especially if there has been any suggestion that something is growing underneath. An inexpensive hatchet or sturdy hoe is helpful in soils heavily laced with tree roots.

Specimens growing in the site examined will either be uncovered or pop out from the raking. It pays to look especially for small, dark, inconspicuous specimens--the larger or more brightly colored ones will then be spotted readily. Once a specimen is found, note the depth and kind of material in which it was growing and look for evidence of associated mycelium. Hypogeous species are often gregarious, and many additional specimens may well be nearby in the same substrate or mycelial system.

Having collected all specimens uncovered at the site, the collector should take notes on their fresh color and odor, on whether latex is exuded from a cut surface, and on whether the specimens change color when either cut or bruised (rub the surface firmly). If no immediate color change occurs, the specimens should be rechecked in 30-60 min and again after several hours.

Specimens can be conveniently kept in waxed sandwich bags (plastic bags are less suitable) until final processing.

Only a few species smell so distinctly and strongly that man can locate them by scent. Europeans have been using dogs and pigs to locate truffle beds for centuries; indeed, animals can be the mycologist's best friends in the search. Most truffles apparently emit odors that animals can detect and most are evidently prized as food by animals. Small rodents seem particularly fond of hypogeous fungi and collect them avidly. Often squirrels will dig them up and set them on tree limbs to dry.

Rodents usually unearth hypogeous fungi one at a time, leaving a small pit; sometimes they will nibble the upper part of a specimen and leave the lower part in place. Usually they will dig only a portion of the specimens present, and often additional ones mature after the rodent's visit. These small pits are among the better clues to the presence of hypogeous fungi; once a pit is found, careful raking of several square feet of surrounding ground at about the same depth as the bottom of the pit is likely to yield specimens. Needles, leaf fragments, and other debris in a rodent pit usually indicate that the specimens matured so long ago as to make further exploration unprofitable. Rodent tunnels usually are not closely related to occurrence of hypogeous fungi; if the bottom of a hole cannot be felt with a finger, it should not be regarded as a clue to finding specimens.

Other animals, too, are attracted by hypogeous fungi. Deer and bear sometimes paw up sizeable tracts that harbor an abundance of specimens. Such areas are profitably searched, and often specimens can be found lying on the surface. In parts of Europe, truffle hunters have regarded clusters of small gnats hovering over the forest floor on warm, calm days as indicators of *Tuber aestivum* and other truffles.

Hypogeous fungi can also be found where the soil is somewhat compacted or where the humus layer is thin. Under such circumstances even relatively small species will hump up the humus enough to be detected by a perceptive searcher, while large species may emerge through the surface. Thus campgrounds, old tractor trails, abandoned roads, and road banks are particularly productive places to look.

As the collector builds experience, he comes to recognize microhabitats likely to produce hypogeous fungi. Generally, they are most numerous and diverse in places with relatively little ground vegetation. Often the soil adjacent to rotten logs is good, particularly the "microbenches" on the upper sides of long-fallen trees lying along the contour of moderate slopes.

Finally, purely random searching can provide supremely fine collections. This is particularly important because seemingly unlikely sites can harbor some species that do not often occur elsewhere, such as the *Hymenogaster alnicola* mentioned above. In one instance, a splendid group of *Tuber* species was discovered during cultivation of a rose garden in a city residential area.

Handling

Careful and detailed notes on fresh specimens are extremely useful. Because the hypogeous mycoflora of much of the world is so poorly known, undescribed species are likely to be encountered. A meticulous description of the fresh characters will greatly enhance the value of a collection if it becomes the type of a new species.

Each specimen should be cut open. Many species resemble each other on the surface but differ in the interior; examining the interior minimizes the chance of including more than one species in a single collection.

Smith and Zeller (1966) have discussed in detail the important characteristics to observe in describing *Rhizopogon* species. These and other features pertinent to hypogeous fungi in general include size, shape, consistency, color and nature of surface and interior, color changes when cut and bruised, reaction of surface and gleba to respective application of solutions of 2.5% KOH and $FeSO_4$, presence and form of a columella, rhizomorphs or mycelial tufts, exudation and color of latex when cut, chambering or venation of gleba, odor and taste. The reaction to KOH and $FeSO_4$ is generally more diagnostically useful with Basidiomycetes than with Ascomycetes, but even in the latter class it can be helpful. Hopefully, other chemicals will be discovered that produce distinctive reactions with some taxa.

The date, location of a collection, plus notes on associated vascular plants, including tree seedlings and shrubs as well as overstory trees, are useful in picturing the specie's ecology.

For several reasons, well-dried specimens are more useful and easier to maintain than those immersed in liquid preservatives. Specimens are preferably described and dried as soon after collection as possible; once deterioration begins, much of their value for later study is lost. This applies particularly to Basidiomycetes, although specimens in very good condition can often be stored in a refrigerator for several days if absolutely necessary. Each specimen should be halved before drying; large ones can be cut in several pieces. This not only speeds drying, but in some species that become hard when dried, it avoids the necessity for taking "heroic" measures later on such as hammering, chiseling, or sawing.

Best preservation results from rapid drying at relatively low temperatures. A circulating oven operated at about 30°C works well. Without good air circulation, specimens will warm up without drying rapidly, and deteriorate rapidly. Silica gel provides a good way for drying specimens in the field (Hoseney, 1963). Small, empty containers (half-pint and one-pint sizes) with lids and a tightly sealed container of oven-dried silica gel can be conveniently taken on collecting trips. When a collection is ready to be dried, the specimens are cut in two or more pieces, depending on size, and covered with the silica gel granules. Pieces should not touch each other. No more than one collection should be put in a given container because, when dried, species can often be difficult to tell apart. One to two days will dry specimens thoroughly by this method if enough silica gel has been added in proportion to the volume of material to be dried.

In special circumstances, e.g., in wilderness areas, where it is not practical to take silica gel, lightweight frames covered with fine mesh screen are useful. The screens can be set up for drying in the breeze and sun or near a campfire. This approach is sufficiently unsatisfactory, however, that it should be used only as a last resort.

Isolation and Culturing

Hypogeous Basidiomycetes would be relatively easy to isolate by tissue culture techniques, could they be depended on to grow in pure culture on known media. As is true of mycorrhizal fungi in general, however, a few grow readily on known media; most do poorly; and some grow not at all. Many *Rhizopogon* species do well on potato glucose agar or on various synthetic media such as Norkrans (1949) agar. *Martellia* species, in contrast, rarely have grown on media tried thus far.

To attempt isolation, a young, freshly collected specimen is cleansed of adhering debris, dried, and broken (not cut) open. Pieces of 2-10 mm across (generally, the larger the better) can be cut out from the interior with a sterile scalpel and placed directly on agar in test tube slants. Since many species are tough, the scalpel blade should be sharp. The tubes can be incubated at room temperature. Hyphae that emerge from the piece of tissue in the first few days, that grow rapidly, or that sporulate are generally contaminants.

Isolation of the Tuberales is much more difficult.

Most species have openings from the surface to the interior, so that the inner tissue usually harbors an abundance of bacteria and microfungi that rapidly overwhelm the tissue from which a culture is attempted. There are several published claims of tissue culture, but none has yet been authenticated. Single-spore culture has been successful in at least one case, but until a variety of species have been tried, no general principles can be established.

Once a hypogeous fungus has been isolated, the culture can usually be kept viable by transferring it every 4-6 weeks if kept at room temperature. Cultures store well at about 5°C; if this can be done, transferring every three months is generally adequate.

See also Parks, 1921; Gilkey, 1954; Hawker, 1954; Singer and Smith, 1960; Trappe, 1962; Smith, 1966; Shaffer, 1968.

SOIL MICROFUNGI

Population Studies

All major classes of fungi are found in dormant or active states in soil; however, on a quantitative basis the Fungi Imperfecti, Ascomycetes, and, in some soils, the Zygomycetes usually comprise the dominant populations. A majority of these species is saprophytic; the remainder may be parasitic or mutualistic.

Fungi generally are most active and, consequently, most numerous in the vicinity of living higher plants and dead organic matter. When living or dead substrata are very limiting or not available, most soil-borne fungi enter dormant phases. This is particularly true of plant-pathogenic species in times when a specifically required host is unavailable or has undergone certain degrees of decomposition. Many plant pathogenic species cannot compete openly with some of the soil-borne saprophytes. Some pathogenic fungi, therefore, are restricted to crop residues in the soil where such species may possess some competitive advantage.

Populations of soil microfungi can vary both qualitatively and quantitatively with changes in seasonal, topographic, and biological factors. Changes or differences in depth or horizon, moisture, temperature, dead or living organic substrata, aeration, and the fertilization, cultivation or chemical treatments of agricultural soils also affect populations, as does the passage of time in relation to the condition of substrata for fungi that occur in succession.

The kinds of vegetative and reproductive structures formed by fungi have a very significant bearing on the methods required for analyses of populations, for isolation and for other studies. Fungi that exist exclusively or for the greatest part of their life cycle as mycelia will often not be detected well by methods that depend primarily on the dispersion and eventual germination of spores or other small propagules. Not all spore-bearing, cellular, or fragmenting forms will be detected equally well by a single method because of differences in the seasonal occurrence, size, viability and source-location of propagules. Population determinations can be complicated by the fact that some fungi produce few detectable propagules per thallus, while thalli of other species individually produce vast numbers of countable units.

Collecting Samples Sites should be chosen and samples taken after consideration of the environmental variables mentioned above. The greatest number of fungi are in the upper 10-20 cm of many soils. The depth at which samples are taken, however, can vary considerably, depending on environmental variables and the purpose of each study. Since populations often are specific to certain ranges of depth (Johnson and Curl, 1972), it is advisable to select critically and to record the particular location or part of the soil profile used. Samples of 200-500 g usually are sufficient. Amounts can be increased depending on the number of physical and chemical tests that are anticipated. Samples can be recovered with a clean trowel or, where one is especially concerned with occurrence relative to edaphic horizons, by a soil tube. Sterile plastic bags are particularly useful as containers in collecting, storing and, if necessary, packaging and shipping. Tags indicating all pertinent information should be attached to the bags at the time of collection.

Handling and Storage The natural moisture content of samples should, to the extent possible, be held constant prior to examination. Relatively wet soils require prompt examination since storage, particularly in sealed containers, promotes establishment of anaerobic conditions that can be toxic to many fungi. Generally, samples should be stored at 4-10°C in a refrigerator or, at least, in the coolest part of the laboratory. In critical population studies, it may be necessary to analyze samples immediately after collection or to include in studies controls that will detect the effects of storage conditions. Various experiments indicate that the most representative assays of natural populations are made by processing samples on the same day as they are collected.

Qualitative and Quantitative Assays Any single method or medium probably will demonstrate the presence of only a portion of the total soil fungi. It is therefore of utmost importance at the onset of a study to determine the kinds of fungi that are to be assayed and, then, to select the materials and methods that will best achieve this.

The direct observation of fungi in soil is very difficult. The general opacity of the soil and the adherence of organisms to particles are major problems. Direct observation has been accomplished by microscopic examinations of vitally stained soil smears, of agar-coated or noncoated glass slides, or of special capillaries that have been buried in soil. Metallurgical microscopes or other instruments incorporating vertical or direct illumination are most useful (Ko, 1971). Menzies (1963) has reviewed other methods.

A more complete analysis of soil-borne populations is usually made via the dilution-plate or soil-plate methods (Warcup, 1950; Johnson and Curl, 1972). The dilution-plate method basically involves the distribution of 0.5 ml of diluted soil suspension over the surface of a solidified agar medium in a standard Petri dish. Plates are incubated at 21-24°C. Resultant colonies are identified and counted. Soil dilutions of 1:1000 or 1:10,000 are often acceptable; but preliminary trials are advisable, since soils can differ considerably with respect to fungus content. Dilutions should be sufficient to yield no more than 40-50 colonies per plate, thereby facilitating optimal germination, growth, and development. Preferably, the agar medium is poured into assay plates far enough in advance so that superficial moisture is lost before plating and, consequently, so that the medium will absorb water added with the sample. This ensures more accurate counts by preventing the movement of propagules in assay plates via free water. An aqueous solution of 0.1% Tween 80 with 0.1% agar can be used in soil dilutions to aid in the suspension of particles and to facilitate the application and adherence of diluted samples to the medium in assay plates. The dilution-plate method is especially recommended for determinations on a wide variety of fungi but is particularly suited to counts of sporulating fungi and may not indicate populations of purely mycelial fungi.

The soil-plate method (Warcup, 1950) in which soil particles are dispersed directly, without previous dilution, in melted, cooled agar medium is often useful in detecting mycelial species, including Basidiomycetes, as well as sporulating fungi. Very small quantities of soil, e.g., 0.01-0.05 g, are stirred into 10 ml of melted, cooled agar medium in each Petri dish. The plates are incubated until colonies develop. This method can be used to demonstrate more accurately the flora associated with particles. The method, however, may result in a poor separation of species and, hence, serve to conceal certain fungi. For this reason, the use of a very selective medium may be mandatory. The soil-plate method may be used alone or, better, as a complement to dilution-plating.

Since the hyphae of some filamentous species adhere to soil particles (Warcup, 1955), washing of soil solids with sterile water prior to soil-plating serves to remove many spores and cellular species and, thus, to facilitate the assay of mycelial forms (Watson, 1960; Johnson and Curl, 1972).

Variations on other techniques involving the insertion of agar, nylon, glass and other materials into soil have been reviewed by Johnson and Curl (1972). These methods generally are more useful for qualitative determinations and for demonstrating or detecting the association of fungi with specific positions in the soil. After recovery from the soil, the previously buried materials are usually incubated on agar media, and resulting colonies are examined and isolated. The more vigorous, rapidly growing fungi are usually first to colonize buried materials such as glass and nylon; materials such as cellulose are nutritionally selective.

Isolation

The direct isolation of fungi from soil and the determination of populations can be expedited through the use of selective media that support the growth of specific groups of fungi. Tsao (1970) has prepared an extensive review of selective media used in isolation.

Control of bacterial growth can be achieved through the individual or combined use of streptomycin, aureomycin, or penicillin at concentrations of 100-300 μg/ml in the medium. Streptomycin alone is usually sufficient and is least expensive. Adjustment of the pH to 4.0-5.0 with added lactic acid discourages the rapid growth of many bacteria; however, this method may bring about greater changes in the growth and appearance of fungi than would occur with the use of selective antibiotics.

Surfactants, such as the nonionic nonyl phenyl polyethylene glycol compounds (Tergitol NPX, J. T. Baker Chemical Co.), can be incorporated into media at low concentrations, e.g., 0.1%, to give additional selectivity against fungi. Detergents and other surface-active agents may promote or prevent germination of fungal spores on dilution plates, or may slow the otherwise rapid mycelial growth of such fungi as *Mucor* and *Trichoderma* (Steiner and Watson, 1965; Lee, 1970). The rapid growth of some species can serve to obscure or prevent the development of other fungi in dilution- or soil-plates. Cationic surfactants generally are more active against fungi than anionic or nonionic forms. Surfactants can simultaneously restrict or prevent growth of some microbial species while encouraging the development of others. In dilution-plating and related procedures, desired selectivity may be achieved by treating soil directly with surfactants prior to plating.

Fruits, seeds, paper, many other natural substrata and a wide array of selective agar-base media containing these materials and their derivatives can be employed in the isolation by soil fungi (Farrow, 1954; Tuite, 1969; Tsao, 1970; Johnson and Curl, 1972). Fruits, seeds, and other plant materials, placed directly in soil, are often good traps for obligate parasites and other fastidious fungi, including many Chytridiomycetes and Oomycetes (Durbin, 1961). General-purpose isolation media include rose bengal medium (M-55) and sodium caseinate agar. Streptomycin is added to these media to prevent bacterial growth. Surfactants should be used only if necessary, since they may be selective or may modify patterns of growth, thereby hindering identification.

Identification

Identification of fungi in dilution plates may be difficult or impossible because of effects on growth and development caused by competition between species, or to components of the selective medium. In such cases, colonies appearing in plates can be coded and the fungi then isolated and grown on media facilitating proper identification. The culture medium can have a pronounced influence on the structures produced by a fungus and thus be a major aid or obstacle to proper identification. This is especially important in classification that is based on the critical morphology of conidiospores, as in the *Fusarium* species. As a general rule, the most representative taxonomic structures are produced on natural substrata or on artificial media containing weak concentrations of nutrients or extracts of host plants. Some fungi may require very specific light, temperature, pH or nutritive conditions for sporulation.

Barron (1968) and Gilman (1957) have prepared manuals with keys to and descriptions of soil microfungi. The manual on Fungi Imperfecti by Barnett and Hunter (1972) is also very useful. Specialized taxonomic treatments are available for many groups of soil fungi.

Soil Fungistasis

Viable spores of many species are unable to germinate in soil under conditions that commonly may be considered favorable for growth. Both biological and chemical factors in soil apparently are responsible for this inhibition. Because fungistasis is an indication of insufficient conditions for the growth of fungi, it appears to be an important mechanism for survival. Lockwood (1964) has reviewed many of the principles and methods involved in studies of fungistasis and concludes that the phenomenon is widespread in natural soils; generally non specific in its inhibitory effects on fungi, although fungi vary in their sensitivity; associated with microbial activity; subject to "annulment" by nutrients; and subject to seasonal variation.

The simplest approaches to the determination of fungistatic properties of soil are: (1) To incubate spores on a cellulose acetate membrane filter, a piece of dialysis tubing or a piece of washed cellophane placed on wet soil in a Petri dish; (2) to incubate spores on sheets of agar medium or other moist, absorbent materials that have been previously placed on or buried in soil in order to extract fungistatic factors (several hours or days may be required for the diffusion of fungistatic substances into the agar that will be used in assays); or (3) to coat glass slides with 1% agar suspensions of spores and to bury the coated slides in soil. Controls can be set up by incubating spores on agar or other substrata treated with or exposed to water only or to sterilized soil. In all cases, the extent of fungistasis is assayed by comparing the percentage germination of spores in the soil treatments vs. controls. Lysis of fungal hyphae, which occurs *after* germination, should not be confused with fungistasis.

The methods for direct observation of soil fungistasis may be more sensitive, but often involve more problems and, hence, have been used less often. These methods include direct observation of spores in soil on glass slides, or the recovery and examination of spores by means of cellulose tape, agar, plastic films, and other adhesive materials after incubation of propagules on soil surfaces. The direct exposure of spores to soil probably gives a more accurate representation of fungistatic potential, since the indirect methods can involve dilution or selective effects, depending on the material used. Furthermore, microbial growth on agar or other materials used in indirect assays can lead to the artificial accumulation of inhibitors.

Demonstrations of the primary importance of microbial activity to fungistasis can be performed by comparing the fungistatic ability of natural soil vs. soil that has been partially or totally sterilized by heat or fumigation. If chemical treatments of soil are used, allow time for the complete escape of residual fumigants.

The counteraction of fungistasis by nutritive materials can be tested by adding low concentrations (e.g., a few µg/ml or more) of various compounds to soil samples or assay media. It is advisable to use a wide selection of test fungi, since individual species often react very differently. Another approach is to expose soil or spores to roots and other plant materials that release nutritive exudates.

DUNG FUNGI

One is virtually assured of finding either Phycomycetes or Fungi Imperfecti on any collection of dung. These, as well as Myxomycetes, Ascomycetes, and Basidiomycetes, may occasionally be found on collections in the field, but it is usually necessary to place dung in a moist chamber for a few days and look for the emergence of particular groups. Most material should be held for two to three weeks to take into account a succession of fungal growth--the Mucorales grow out the second and third day, followed closely by Pyrenomycetes and Discomycetes, and lastly by Basidiomycetes. Dung collected too soon after excretion is often overrun by bacteria but is less likely to be troublesome if air-dried for a day or two. Dung of certain livestock, such as cattle, remains moist under a thin outer layer for long periods even in an extremely dry climate and consequently produces large crops of fungi even if collected long after excretion. Dung that has been exposed several times to rain yields very little fungus growth other than by air or soilborne organisms. Dried collections may continue to yield fungi for several months (up to four years) when again placed under moist conditions.

There is very little information on the seasonal occurrence of most coprophiles. There is evidence that fewer Pyrenomycetes and Discomycetes are found during extremely cold or dry seasons, but in any season one can usually find at least some representatives of the major fungal groups when the dung is placed in a moist chamber at room temperature. Records indicate that most of the coprophilic genera are distributed throughout the world. However, many species are restricted to the dung of a particular animal or group of animals, a large majority to that of herbivores. By far the greatest variety of fungi have been reported on cow dung, horse dung, and rabbit dung, perhaps only because these are the kinds most frequently collected. Likewise a number of organisms seem rather restricted, such as *Lasiobolus cainii*, a multispored species found only on porcupine dung; *Trichobolus zukalii*, only on certain ruminant dung; *Podosordaria leporina*, on rabbit dung; and *Ascozonus* spp., largely on dung of small rodents.

Dung may be collected in small paper bags, waxed paper, glass dishes, or in any available container of suitable size. If the material is not to be examined immediately or soon placed in the moist chamber, it will be necessary to dry it thoroughly to keep down bacterial contamination and reduce the insect problem. In any event, if the dung is not incubated within a few days, some type of insect control will be necessary. Paradichlorobenzene is very effective and does not seem to influence fungus growth, at least for a few days; the effect over a long period of time has not been studied.

Before placing the dung collections in the moist chamber, they should be checked for organisms that may have grown out in the field. Petri dishes floored with moistened filter paper or absorbent towels are very useful for small samples, larger dishes for other collections. Dry dung should be dipped in water for a few minutes and then placed in the moist chamber. Moist chambers should be kept near a window and at room temperature. The material should be examined with a dissecting microscope. Lids of the dishes may also be inspected for evidence of ejected spores. If so, lids may be replaced with sterile ones, often permitting one to obtain uncontaminated spores.

Cultures may be obtained in a number of ways. Pure cultures of Mucorales can usually be obtained by removing spores or small amounts of mycelial growth from the dung. Since spores of the Ascomycetes and some of the Zygomycetes are forcibly ejected, they can often be collected for mounting or for culturing by placing sterile cover slips, held in flamed clips, above the sporulating fungi. Small fragments of dung that bear Pyrenomycetes or Discomycetes can be attached to the under surface of Petri dish lids, thus allowing the spores to be shot onto the medium below.

Myxomycetes

Myxomycetes frequently emerge on dung although less regularly than do the higher fungi. Among these are species of *Physarum*, *Didymium*, *Perichaena*, and *Comatricha*. The beauty of these organisms rarely escapes those who are searching for other coprophilic forms.

Phycomycetes

Many interesting Zygomycetes appear on dung. Common among the Mucorales are *Phycomyces*, *Circinella*, and *Mucor* of the Mucoraceae; *Cokeromyces*, *Radiomyces*, and *Thamnidium* of the Thamnidiaceae; *Kickxella* and *Coemansia* of the Kickxellaceae; *Dimargaris* and *Dispira* of the Dimargaritaceae; *Syncephalastrum* of the Syncephalastraceae; *Piptocephalis* and *Syncephalis* of the Piptocephalaceae; *Endogone* of the Endogonaceae; and *Choanephora* of the Choanephoraceae. *Dimargaris* and *Dispira* are parasitic on other Mucorales, rarely on *Chaetomium*.

Phycomyces has been studied more extensively from a physiological standpoint than any other genus in the order, as evidenced by the publication of at least 150 papers. The positively phototrophic sporangiophores are larger and longer than those of any other known fungi. Because of the large size of gametangia, species of *Phycomyces* are excellent organisms to demonstrate zygospore formation. Sporulation and zygospore formation occur readily on asparagine glucose agar (M-81), or on malt agar, or on potato glucose agar. Young colonies grown on sterile squares of dialysis tubing placed on the surface of agar media in Petri dishes or culture tubes may be prepared with minimum effort for microscopic observation.

"Water Jet" mechanism of spore discharge in *Pilobolus*, found on several kinds of dung but most commonly on horse dung, can be demonstrated in moist chambers. The forcible discharge of the mature sporangium of *Pilobolus* is a dramatic and characteristic feature of the asexual reproduction in this genus. The structure of the sporangiophore and the discharge of the sporangium were described by Buller, and recent improvements in techniques (Page, 1964) confirm his theory that the sporangium is propelled by a jet of cell sap that breaks into droplets. (See also Chapter 22.)

A diurnal cycle is also evident in *Pilobolus*, sporangiophores developing in late afternoon and evening, maturing through the night and morning, discharging sporangia often around mid-day, another crop of sporangia forming in late afternoon. As a result of nutritional studies, it is now possible to grow *Pilobolus* on medium of known chemical composition (M-78) and observe the effect of alteration of chemical as well as physical factors in the environment. Asexual reproduction may be checked on cultures grown in the dark for three days at 25°C and then exposed to light. Zygospores will form under similar conditions when compatible strains are mated.

Basidiobolus, a genus of saprobic Entomophthoraceae, is commonly encountered in excreta of frogs and lizards, intestinal contents of salamanders, and in humus. All species grow readily in pure culture on various media such as nonsupplemented cornmeal agar. Even in young cultures of *B. ranarum* and *B. meristosporus*, asexual and sexual reproduction usually proceed concurrently. The rocket-like action by which the expanded distal portion of its conidiophore propels the globose conidium is described by Dreschler (1956), as are details for collecting and isolating these fungi. At temperatures near 20°C, a colony of *Basidiobolus*, on attaining a diameter of several millimeters, usually begins to produce both conidia and zygospores. Phototropic response of conidiophores accelerates growth toward the light by forcibly discharged conidia that germinate to produce new phototropic conidiophores. In zygospore formation, conjugation takes place between adjacent hyphal bodies.

Ascomycetes

Gymnoascaceae Among the simpler Ascomycetes found on dung are members of the Gymnoascaceae. *Arachniotus*, *Gymnoascus*, and *Myxotrichum* are genera that have coprophilic species found commonly on dung of small rodents. All should prove good for demonstrating cleistothecial type ascocarps with a plectomycete centrum, i.e., one in which the asci are borne at different levels and deliquesce before maturation, leaving ascospores scattered loosely throughout. Papers by Kuehn and Orr (1964) and Orr *et al.* (1963b) will be valuable for those desiring a closer study of these organisms.

Isolations of Gymnoascaceae from dung may be accomplished by direct transfer of ascospores or asexual spores from sporulating material onto Sabouraud's agar fortified with 10,000 units/ml penicillin, 100 units/ml streptomycin, and 0.5-2.0 mg/ml actidione. Sexual reproduction may be induced on dung-decoction-oatmeal agar prepared as follows:

- Dry about 250 g shredded dung of various sorts; steep in 1 liter of tap water for 12 hours and filter through four alternating layers each of gauze or cheesecloth and cotton.
- Bring the decoction to 2 liters with distilled water and sterilize in 1-liter flasks.
- Place 40 g of rolled oats in 1 liter of tap water and steam in an autoclave for 12 min at 10 lb pressure.
- Filter this oat decoction through four layers of fine-mesh cheesecloth, and bring to 2 liters with distilled water; sterilize in 1-liter flasks.

- Add 20 g unpurified agar to a liter of 50% mixture of each of the above solutions and, after autoclaving, pour into Petri dishes. A cellophane strip, as described above, is useful in studying developing stages.

Chaetomiaceae Species of *Chaetomium* and *Lophotrichus* of the Chaetomiaceae, which are mainly cellulose-destroying fungi, are commonly found on dung of herbivorous animals. These are especially easy to obtain in culture and provide good material for the study of crozier formation of asci and the development of perithecia. As in the Gymnoascaceae, asci deliquesce before maturity, but a distinct layer or hymenium of asci is formed in this group. Students will find papers by Ames (1963) and Whiteside (1962) useful in studying this group.

Pyrenomycetes Most common of the Pyrenomycetes found on dung are the Sordariaceae. Seldom does one find a collection of dung that will not bear species of *Sordaria*, *Delitschia*, *Sporormia*, *Bombardia*, *Coniochaeta*, *Zygospermum*, or *Gelasinospora*. The positive heliotropic curvatures of the perithecial necks of many of the Sordariaceae are admirably adapted to promote efficient liberation of spores from reproductive organs that are produced on an irregularly disposed substratum. A number of very interesting physiological and genetic studies have been performed with members of the Sordariaceae.

Many of the Sordariaceae are homothallic and form perithecia from single, haploid ascospores. Certain species of *Gelasinospora* are heterothallic. When two isolates of opposite compatibility are mated on a suitable medium, dark, pyriform perithecia containing asci with eight uniseriately arranged ascospores are produced. From the cytological study of ascospore formation it is evident that this linear arrangement of the ascospores indicates the exact order of nuclear movement during meiosis. Therefore, an analysis of the tetrads yields information about the segregation of alleles during meiosis. Satisfactory perithecia for the dissection of asci may be obtained in culture within two or three weeks. Successful fruiting may be obtained on several standard media, cornmeal agar being the most frequently employed. If cultures are held too long, a majority of ripe asci will eject their spores. Mature asci may be dissected freehand, employing fine glass needles and a dissecting microscope. Care must be taken to retain the exact order of the ascospores as they are removed from the ascus. To stimulate germination, spores may be placed on cornmeal agar containing 1:100,000 distilled furfural and heat-treated at 60°C for a half-hour. After germination, spores with short germ tubes may be transferred to agar slants or dishes for incubation. From this point on, various crosses may be made and genetic data analyzed in a number of ways.

Many species of *Sordaria* grow and fruit well on Difco cornmeal glucose agar with 0.1% yeast extract. Spores collected from material brought in from the field or from perithecia in culture germinate well on 0.7% sodium acetate agar. When mycelium is about four days old, many protoperithecia appear beneath the agar and microconidia of heterothallic species begin to appear on upright, branched conidiophores. Excellent perithecial production may be obtained in heterothallic forms either by spermatization or by pairing compatible mycelia on agar plates. Ascus analysis, similar to that discussed above for *Gelasinospora*, may be carried out.

Other Pyrenomycetes such as species of *Nectria*, *Xylaria*, *Poronia* (= *Podosordaria*), and *Hypocrea* have been reported on dung, but only *Podosordaria* with any regularity. *Podosordaria leporina* is commonly to be found on rabbit dung. This is a good organism for illustrating perithecia embedded in a superficial fungal stroma, a characteristic feature of the Xylariaceae.

Loculoascomycetes It has been clearly shown (Morisset, 1963) that *Sporormia leporina* and similar species are members of the Loculoascomycetes, not Pyrenomycetes. Such species make excellent examples to demonstrate loculo-stromatic ascocarps with bitunicate asci. Ascocarps of *Sporormia* are not initiated by an ascogonial coil as is true of the Sordariaceae, but instead certain privileged cells of a hypha enlarge and divide in three planes to form a young pseudothecium, inside which asci form and lyse a cavity.

Discomycetes A very large number of Discomycetes are coprophilic; most have been placed until recently in the Ascobolaceae of the Pezizales. More prominent among these are species of *Ascobolus*, *Ascodemis*, *Ascophanus* (= *Iodophanus*), *Ascozonus*, *Cheilymenia*, *Coprobia*, *Coprotus*, *Fimaria*, *Humaria*, *Lachnea*, *Lasiobolus*, *Peziza*, *Saccobolus*, *Thecotheus*, *Thelebolus*, and *Trichobolus*. Most of the Discomycetes appear on dung three or four days after rainfall in the field. Approximately the same time interval is necessary for mature apothecia to develop in a moist chamber. It is obvious that the life cycles of many depend on the dispersal of spores by animals that consume plants onto which spores have been ejected. That spores are forcibly ejected by almost all of the Discomycetes enables one to obtain pure cultures with relative ease, except in a few cases where spore germination presents difficulties. Spores of *Ascobolus* and *Saccobolus* are arranged in the ascus in one or two rows or irregularly disposed, and are held firmly together. Spores of *Ascobolus* and other genera are held by a gelatinous sheath, but in *Saccobolus* by an additional membrane-bounded cytoplasm that hardens around the group of spores, fusing them into a packet of eight. In both cases, spores of an ascus are ejected as a unit and usually for a greater distance than if they were shot individually. If one observes closely, he will find the asci of many of the Ascobolaceae are heliotropic. By rotating culture dishes in reference to the light source, one can note, after a period of growth in a certain position, the bending of ascal tips toward the light. This heliotropic response is aided greatly in several genera by the protrusion of asci beyond the hymenial surface. The diurnal periodicity in the ripening of asci is such that each morning many asci go through their final phases of stretching and discharge spores almost simultaneously about midday or in early afternoon. After one series of asci has exploded, another (which will discharge its spores on the following day) immediately begins to develop. We thus see that the protrusion of ripe asci, their periodic development, and their heliotropism are intimately correlated with one another. Apothecia also orient themselves such that ascal tips are toward the light.

In some forms, spores germinate directly on water or routine fungal media, but in most cases pretreatment is necessary. Spores of *Ascobolus* and *Saccobolus*, although found difficult to work with by some, can be stimulated to germinate by adding a drop or two of 2% KOH over the ejected spores and incubating overnight at 35°C. Frequently, spores will begin to swell after about six hours and many germinate after 12-15 hours.

Dasyobolus immersus has a periodic growth pattern due to branching and sub-branching at the leading edge. The linear growth rate is constant; only the branching pattern is rhythmic. The time period necessary for the formation of each band is temperature dependent, light independent, and chemically controllable. Persons interested in employing this or similar organisms in the study of biological rhythms should refer to the work of Berliner and Neurath (1965).

Ascocarp Development In most species of coprophilous Discomycetes thus far investigated there is a conspicuous multicellular ascogonial coil, the central parts of which give rise to ascogenous hyphae. *Ascobolus furfuraceus* is one of the commonest dung species; the ascocarp is green or brown and has a characteristic scurfy margin. Sometimes the ascogonial coil consists of as many as 20 cells. Bistis and Raper (1963) found in the sexual reaction of *A. stercorarius*, a heterothallic species, that the initial reactants are a mature vegetative mycelium (the ascogonial parent) of one mating type and potential fertilizing elements of the opposite class. Vegetative cells capable of differentiation into fertilizing elements include nongerminated oidia, oidial germ tubes, severed hyphal tips, and germ tubes of ascospores. Four-day-old mycelium is usually considered mature mycelium. Ascogonia usually begin as short, one- or two-celled, club-shaped branches growing in an erect or oblique position at the surface of the medium. They are commonly associated in pairs or groups of three or four. However, in homothallic species they usually occur single.

A slide culture technique (Riddell, 1950) is very useful in studying the process of plasmogamy and early ascocarp formation. For this technique one employs as moist chambers Petri dishes floored with one or two pieces of filter paper and with a small "U" tube on which to rest a microscope slide (Figure 1). Sterilize the moist chamber, including a slide and cover slip. With a sterile scalpel, remove a square of agar on which the fungus is growing and transfer it aseptically to the slide inside the moist chamber and cover it with the sterile cover slip. If the agar is thin and the squares are cut at least 5 mm narrower than the cover slip, contamination around the edge is a minor problem, even when removing and returning the slide for periods of observation. This technique can be applied just as well to the observation of spore germination.

Discharged ascospores of any species of *Ascodesmis* will readily start germinating within a few hours in a moist environment. Immersed in any liquid, however, the spores do not germinate. Dried spores can be revived even after a period of many years. Fast growth, abundant aerial mycelium, and sexual reproductive structures are plentiful on modified Leonian's agar (soluble starch, 10 g; yeast extract, 5 g; KH_2PO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; agar, 20 g; tap water, 1000 ml). The mycelium spreads rapidly from the point of inoculation. Abundant but scattered apothecia form after one or two days and mature ascospores appear after three to six days. Production of apothecia in pure cultures slowly decreases after many transfers. Unlike many other coprophilous Ascomycetes, light is not required for either formation or maturation of ascospores of *Ascodesmis*. Because of the lack of an excipulum, species of *Ascodesmis* seem to be very suitable for cytological work. It is very difficult to demonstrate fusion of gametangia convincingly, however, because all cells are multinucleate and the coiled hyphal pairs occur in dense clusters in which cell walls seem to disintegrate.

Coprophilous Discomycetes display great variation in the manner of ascocarp development, ranging from gymnocarpic, pseudoangiocarpic, hemiangiocarpic, to angiocarpic. Most species of *Coprotus* (Kimbrough and Korf, 1967) are gymnocarpic, i.e., their asci are exposed throughout ascocarp development. Spores of many of these species will germinate on dung decoction agar or on a 2% agar prepared from fluid in which dung has soaked for a few hours (approximately 10 g of dung per 100 ml water has proven sufficient). Spores may be shot directly onto the medium or onto water agar and single spores then transferred to the second medium for germination. After germination, spores are then transferred to a medium for growth (dung-oatmeal or Leonian's agar is recommended). Processes of plasmogamy, fertilization, and ascocarp development may be checked periodically. Since apothecia of this genus are very small, dehydrated and fresh apothecia should be embedded in 5% water agar and frozen in a few drops of 50% aqueous (V/V) mucilage and then sectioned longitudinally on a freezing microtome. Frozen sections should show asci and apothecia in various stages of development.

Coprotus sexdecimsporus and a few other species have asci exposed early in development. However, accelerated extra-excipular hyphae soon grow up and around the asci, making the development appear angiocarpic or closed. This pseudoangiocarpic pattern may be checked also with the procedures described above for *Coprotus* spp.

Iodophanus (Ascophanus) carneus, which is very common on cow dung, has a hemiangiocarpic development. The ascogenous hyphae and young asci are completely enclosed by the excipulum. About the time of spore maturation, however, the area above the asci becomes torn by expanding asci and the apothecium quickly becomes cupulate. Successful spore germination and growth may be obtained with the technique described above for *Coprotus*.

Very little is known about sexuality and development of most of the coprophilous Discomycetes. Most of the species investigated are homothallic, thus making it easier to obtain successful fruiting in culture. The difficulty of spore germination and production of apothecia in culture of many species has left many gaps in our knowledge of the genetics and physiology of this group.

Basidiomycetes

Basidiomycetes most commonly found on dung are species of *Coprinus*, *Stropharia*, *Galera*, and *Panaeolus* of the Agaricales, and *Cyathus*, *Crucibulum*, and *Sphaerobolus* of the Nidulariales.

When dung, but in particular that from horses, is placed inside a moist chamber and left in diffuse daylight at room temperature, species of *Coprinus* will grow out in 9-10 days with surprising regularity. They are adapted to their environment in that the mycelium utilizes materials contained in dung, the spores are able to pass uninjured through the alimentary canal of herbivorous animals, and the fruit bodies are well suited to the environment. Young sporocarps of *Coprinus*, although they may initially be phototropic, become negatively geotropic and the pilei orient themselves in such a position that they expand and shed their spores into the air. This response is constant, regardless of the direction of light. In most species of *Coprinus* a wave of basidial maturation proceeds centripetally from the periphery, closely followed by the deliquescence (autodigestion) of the cap; the drops of ink-black fluid that fall from the liquefying cap owe much of their color to the large numbers of spores they contain. Indeed, the group is called the "inky-cap mushrooms."

In species of *Coprinus* it is very easy to observe the discharge of spores from the basidia near the edge of a "deliquescing" gill, provided the gill is laid flat in a van Tieghem cell to prevent too rapid loss of water. With the low power of the microscope one can then observe large numbers of basidia actively shedding their spores. Four spores of a basidium are shot off from their sterigmata, usually one by one, in the course of one or a few minutes. In nature there is little doubt that some of the smaller and more delicate species of *Coprinus* are largely dependent on the weather for success in liberating spores. In very dry conditions the pileus will often dry and shrivel before discharge.

Transfers of tissue from the stipe of young sporophores quickly grow on potato glucose agar plus 0.1% peptone, phytone, or coconut milk. Asheshov's A 58 medium (M-16) (Routien and Simonzi, 1960) supported sporocarps when the fungus was grown in 300-ml Erlenmeyer flasks on 75 ml of agar. Most of the species investigated are homothallic. The genetics of sexuality in *Coprinus* has been widely investigated, as evidenced by Raper's (1966) extensive treatment and bibliography in his study on sexuality of higher fungi. *Coprinus* has been studied in a wide range of physiological activities. Among those are thiamine deficiency; temperature requirements, carbon source, and optimum pH; spore germination; nitrogen requirements. It is evident from a number of studies that species of *Coprinus* will not produce sporocarps in the absence of light. However only brief illumination is necessary for fruitbody development.

Species of *Panaeolus*, which frequently occur on dung, may cause a temporary paralysis or intoxication similar to alcoholic intoxication. They are said to be one of the components of the "witches brew" of tribes of Latin American Indians. Those interested in sporocarp development will find *Coprinus* and *Panaeolus* good for demonstrating the hemiangiocarpic type. Mottling of the gills is one of the most obvious field characters of *Panaeolus*. Unlike *Coprinus*, spore discharge may last for several days. Species of *Panaeolus* have been used in such physiological studies as tryptophan hydroxylase activity, psychoneurophysiologic effect, and alcohol sensitization. Those interested in the genetics of sexuality may refer to Vanderdries (1924).

The genus *Stropharia* contains some of the commonest of field mushrooms. They are coprophilous and grow on horse dung through Europe and North America. When material is placed inside a moist chamber, sporophores develop usually within two weeks. Buller showed that the mycelium retains its viability in the dried condition for at least five months and probably longer. The very interesting *S. epimyces* is parasitic on coprophilic species of *Coprinus*. Species of *Stropharia* are excellent for demonstrating a viscid or glutinous universal veil. It is evident that light plays an important part in sporophore production in *Stropharia* and other coprophilic agarics. Sporophores always arise from beneath or from darkened crevices of the substrate. They should prove to be very interesting organisms to study the influence of light on agaric fruiting.

While very few fungi are found on old dung that has withstood a number of rainfalls, members of the Nidulariales or "bird's nest fungi" are exceptions. *Sphaerobolus* is most frequently found on stumps, sticks, boards, and woody substrates. It is not only lignicolous but also coprophilous. Even though species have been reported on a number of types of animal dungs, it appears most frequently on that of horses, cows, and rabbits. It requires approximately three weeks for sporocarps to appear on material placed in moist chambers, after most other fungi have come and gone. According to Buller, the dry discharged gleba of *Sphaerobolus* may remain viable for upwards of ten years. When placed in water, their gemmae send out a profuse, radiating, clamp-bearing mycelium. Buller describes many aspects of these remarkable fungi, including their structure, development, spore discharge, ecology, and relationship to other fungi.

In the Nidulariaceae, or true bird's nest fungi, several peridioles are formed in each sporocarp. At first connected in a continuous gleba, the peridioles early become separated from each other and lie free in the cavity or are connected to the peridium by long slender strands, the funiculi. Species of *Cyathus* and *Crucibulum* are commonly found on very old dung. Brodie (1962) states that if peridioles are macerated and the spore suspension obtained is held at 40°C for two days, some spores usually germinate. *Cyathus stercoreus* and other species fruit on solid or liquid medium (nutrient agar commonly used) in 40 days without much trouble. Some species have never been induced to fruit. The basidiospores of the Nidulariaceae lie freed from their basidia and are all thoroughly mixed in the peridiole when the latter is mature. Thus there seems to be no way of isolating the four spores that together would represent a tetrad. Without the opportunity for tetrad analysis, certain kinds of genetic work are difficult. The most extensive work in this area is that of Fulton (1950).

See also Brodie, 1951; Larsen, 1971.

PYROPHILOUS FUNGI

Burnt ground and bonfire sites provide a habitat for a number of fungi, some of which are virtually confined to such places. In the tropics the most obvious is *Neurospora*, but in temperate latitudes the flora is largely dominated by operculate Discomycetes (and their conidial states) and a number of such characteristic

agarics as *Flammula carbonaria* and *Omphalia maura*.
See also El Abyad and Webster, 1968a, b.

Collecting

Burnt soil is very crumbly when dry and care has, therefore, to be taken when collecting specimens. Flat, airtight tobacco tins with snap-on lids have been found most suitable. The tin should be pressed down over the soil to mark out an area of the soil that will exactly fill the tin. The outline of the tin should then be cut out with a knife and the slab of soil gently lowered into place. An alternative technique is to cut out a slab of soil and place it on a shallow food tray and then pull a polythene bag over it; the tray should be held horizontally while being returned to the laboratory, preferably in a basket. Apothecia of species growing on charred wood are best removed with a saw and the wood wrapped in a polythene bag.

Isolation

Discomycetes can be isolated by allowing the ascospores to be projected onto an agar surface. Germination of most species occurs readily on 2% malt extract agar, but this should be filtered before sterilization to ensure that it is transparent. Have an empty dish of the same size available. Place a small piece of moist folded filter paper in the lid of the empty dish, preferably eccentrically. Place a small piece of burnt soil or charred wood bearing apothecia on the filter paper. Exchange the lids of the two dishes so that the malt agar is now over the apothecia. Place the dish on a windowsill so that the apothecia are illuminated from above, which will encourage the discharge of spores onto the agar. The upper part of the dish containing the agar can be rotated at intervals to ensure an even deposit of spores. After 24 hours, or when sufficient spores have been discharged, carefully replace the base of the dish bearing apothecia with a sterile lid. Contamination from bacteria can be minimized by the addition of antibiotics to the agar before pouring the plate.

Germination

The spores of most pyrophilous Discomycetes germinate without special treatment, but the following germinate best after heat treatment as indicated below:

	Temperature	Time
Anthracobia maurilabra	50°C	10-15 min
Anthracobia melaloma	50°C	5-15 min
Ascobolus carbonarius	50-70°C	2-15 min
Peziza anthracophila	50°C	2-15 min
Peziza praetervisa	50°C	2-10 min
Trichophaea abundans	50°C	2-5 min

In some cases, e.g. *Trichophaea hemisphaerioides*, heat-treated ascospores germinate less well than untreated spores.

In most cases spores germinate sufficiently for transfer of sporelings after 24 hours. For single-spore cultures, well-separated spores should be marked by an India ink dot on the Petri dish. A small block of agar containing the single germinated spore can be transferred by sterile needle to other media.

Cultures

Cultures can be established on agar media or on sterilized burnt or unburnt soil.

Burnt soil is collected in bulk in the field. Fruiting cultures can be established by placing 60 g burnt soil in 9-cm crystallizing dishes covered with a Petri dish lid, and autoclaving at 15 lb for 15 min. The burnt soil can be inoculated from a suspension of ascospores obtained by crushing an apothecium in sterile water, or by a slab of agar from a pure culture. Light is essential for the fruiting of many of these fungi, and it is helpful to maintain soil cultures in a greenhouse.

The table below (Table 7) lists the fungi that have been induced to fruit in culture and, in several species, the agar media on which fruiting occurred.

Stock cultures can be maintained on slants of the appropriate medium, but in many cases such cultures eventually lose their ability to fruit. Since ascospores

TABLE 7 Pyrophilous Discomycetes Induced to Fruit in Culture

Species	Homothallic or Heterothallic	Sterilized Burnt Soil	Agar Medium	Time Taken to Fruit
Anthracobia macrocystis	Hetero		2% Malt Extract Agar	
Anthracobia melaloma	Homo	+	2% Malt Extract Agar or Olive's*	14 days
Ascobolus carbonarius	Hetero	+	-	21-28 days
Peziza anthracophila	?	+	-	25 days
Peziza praetervisa	?	+	-	25 days
Pyronema domesticum	Homo	+	-	4 days
Trichophaea abundans	Homo	+	Medium M**	7 days

*Olive, 1950
**Gwynne-Vaughan and Williamson, 1927

may retain their viability for periods of up to two years when stored on dry Petri dish lids, it is often preferable to maintain cultures as deposits thrown onto the surface of the lids. An alternative technique is to store the spores in sterilized dried soil by drawing air from a fruiting culture growing in a Petri dish or crystallizing dish through a filter funnel and a tube of sterilized dry soil (see Figure 11).

Conidial States

A number of the Discomycetes from burnt soil have characteristic conidial states. These include *Peziza anthracophila* and *P. praetervisa* (*Oedocephalum* states), *Peziza atrovinosa* (*Ostracoderma* state), and *Trichophaea abundans* (*Botrytis* state).

Conidia can also be stored in air-dried soil.

The tubes containing dried soil and spores should be plugged at each end with cotton and stored at room temperature. They will retain viability for two years or more when ascospores are used. To revive such cultures, push out the soil and sprinkle it over the surface of sterilized burnt soil in a crystallizing dish; place the dish in the light. If necessary, add sterile water to the dish.

Special Techniques

Pyronema domesticum This is an excellent fungus for demonstrating the large ascogonium, trichogyne and

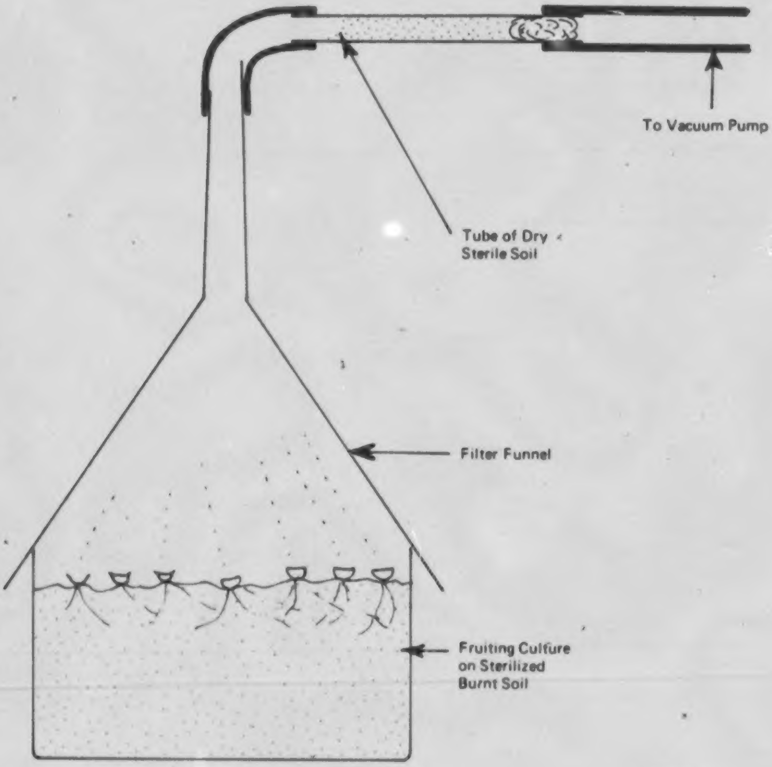
To Vacuum Pump
Tube of Dry Sterile Soil
Filter Funnel
Fruiting Culture on Sterilized Burnt Soil

FIGURE 11 *Apparatus for preparing spores for storage in sterilized dried soil.*

antheridia. Preparations of the young sexual stages can be made by lifting off the rudiments with a fine sewing needle held in a chuck handle. Clean preparations are facilitated if a few small pieces of broken clay pot are pressed onto the surface of burnt soil in the crystallizing dish before sterilization. Two to three days after inoculation, if incubated in the light, the minute pale pink primordia will appear. The ascogonia should be mounted in cotton-blue lactic acid. Temporary preparations are superior to permanent ones for demonstrating gross morphology.

Trichophaea abundans The very rapid growth and conidial production by this fungus make it a troublesome contaminant, every bit as troublesome as *Neurospora*! Unless there is good reason for wishing to demonstrate this fungus alive to the class, one is well-advised to kill the conidia by exposing Petri dishes to formalin vapor in a closed container.

THERMOPHILIC FUNGI

Although most forms of thermophilic fungi appear to be widely distributed and may be isolated from a wide variety of plant materials, including hay, grass, peat, leaf mold, nesting materials, compost, mushroom beds, trash decomposition piles, and from the dung of several herbivores, the investigator interested in securing representative thermophiles for general laboratory study is most likely to achieve success by concentrating on a few naturally-occurring substrata such as horse dung and garden compost. However, if other types of herbivore dung and other microbially self-heated plant matter, such as mushroom compost, are readily available, they should not be entirely excluded.

In general, better results can be expected if the substrata selected are relatively fresh, which insures adequate moisture and nutrients for the developing organisms and freedom from a heavy growth of mesophilic organisms. In some cases thermophiles may appear on such thoroughly dried materials as hay or straw or even dung that have been rehydrated. Fresh material that cannot be examined immediately may be stored under normal refrigeration in plastic bags or covered dishes until ready for use.

Whether the collected material is to be used directly for laboratory study or as a source of fungus inoculum for the establishment of agar cultures, the method of handling the natural substrata is the same. This consists of placing a portion of moist dung or small pieces of plant material in crystallizing dishes or other suitable covered glass containers that have been previously lined with several thicknesses of moist paper toweling. The dishes should then be placed in an incubator and held for four or five days at a temperature of about 50°C. It is important that the incubation temperature be relatively high, for lower temperatures will encourage the growth of ubiquitous saprophytic molds that will soon overrun the substratum and that will make isolation of thermophilic fungi impossible.

To verify the presence of thermophilic fungi on the material, the dishes should be examined, with the lids removed, under a dissecting microscope. Discernible hyphae, spores, and such fruiting bodies as cleistothecia or perithecia can then be carefully removed with fine, sterile forceps or needles to a slide for direct observation.

Because the thermophilic fungi are easily established and maintained on agar plates or slants and because they sporulate typically and form typical fruiting structures on laboratory media, it is far more practical to establish a pure culture of each organism, once the gross culture has been obtained upon natural substrata, than to attempt each time to use natural material for demonstration.

Isolation and Culture

For the original isolation and for the maintenance of subsequent pure cultures, several types of agar media may be employed. Those media that have proven to be most satisfactory are yeast-starch agar (M-70a), yeast-glucose agar, peptone-glucose agar (M-52), oatmeal agar, and Czapek's agar. On the other hand, some of the more commonly used media, such as nutrient agar and potato glucose agar, are not recommended since both tend to dry out rapidly at higher temperatures. With few exceptions, the thermophilic fungi grow well on yeast-starch agar; it is recommended that this substratum be used both for isolation of the fungi and for maintaining stock cultures.

There are a large number of thermophilic and thermotolerant bacteria whose growth must be circumvented in order to successfully obtain unifungal cultures. The addition of antibiotics to the agar medium will prevent bacterial growth. Eight hundred µg/ml of aureomycin and 30,000 units of streptomycin have been successfully used for this purpose.

For plate cultures, small size Petri dishes (6-cm diameter) should be used if possible. Not only are they easier to handle and store than are standard size dishes, but the smaller surface area reduces the rate of desiccation of the agar at the high temperatures employed.

Precautions should be observed to insure that the materials and cultures do not dry out during incubation. If, however, Petri dishes are wrapped tightly in plastic or waxed paper, rapid evaporation of water from the agar with subsequent condensation on lids will result in an extremely rapid spread of bacteria in a culture.

Another precaution is that daily observation, or even 12-hour observations, should be made. The thermophiles grow surprisingly rapidly as compared with mesophiles. Early observation and early transfer of unifungal growths into test tubes is recommended.

In determining growth responses to temperature, it is wise to provide heat shields (Kirk, 1969), consisting of corrugated paper disks wrapped in metal foil, below each stack of Petri dishes.

The manner of growth of thermophiles on dung, straw or other substrata does not differ markedly from other fungi, hence the techniques used for transferring the growth to agar plates or slants are more or less standard. Spores of the imperfect forms may be transferred to the media by means of a sterile, straight inoculating needle. But because the growth on dung or other material may not be particularly heavy and because many of the spores are rather dry and do not always readily adhere to the needle, it is suggested that the flamed needle be first touched to the sterile agar in a dish or test tube and then carefully applied (while observing the material under a dissecting microscope) to the fungus growth. The slightly moistened needle increases the likelihood of removing the desired spores and at the same time reduces to a minimum the disturbance or destruction of the mycelial growth.

After the primary transfer plates have been inoculated, they should be incubated at 50°C until a good growth is established that, in most cases, should occur within two or three days. The plates should then be removed to a 40°C incubator for the remainder of the incubation period in order to prevent excessive loss of moisture. Because of the relatively high initial temperature used, which rapidly dries out and commonly causes a splitting of the agar, it is necessary to place the inoculated plates in a large, glass moist-chamber lined with damp toweling before incubating. Also, it is imperative that the plates be placed in an inverted position in the moist chamber. It is also helpful to place a piece of dry toweling over the plates before replacing the moist-chamber lid. This prevents the condensed moisture on the under surface of the lid from dripping directly onto the inverted plates. Since most of the imperfect thermophiles normally produce mature asexual spores after two or three days at 40-50°C, they should be examined at this time in order to observe best the attachment of the spores on the conidiophores. Similarly, the two known thermophilic species of the genus *Mucor* produce mature sporangia with sporangiospores in this relatively short period of time; they too should be examined early. However, if the observer wishes to study the zygospores produced by these species, the cultures should be allowed to incubate for an additional day or two. The zygospores occur on the prostrate hyphae; hence the dense, erect sporangiophores should be pushed aside with forceps or needles so that hyphal material can be removed directly from the surface of the agar. It may even be necessary to remove a thin layer of the agar in order to obtain the zygospores. The remaining known thermophiles produce various types of ascocarps with the contained asci and ascospores, and these structures require somewhat more time to mature.

In general, an incubation period of at least six or seven days should be allowed for development of mature ascospores. In some of the ascomycetous forms, better results can be expected if the material is examined after ten days or more. Of course, if the observer wishes to study the stages of development of the asci and ascocarps, then representative ascocarps should be removed at daily intervals beginning at about the fifth or sixth day. If the larger, standard size Petri dishes are used and the culture is allowed to incubate for several days, ascocarps in various stages of development may be obtained from the same plate. Mature structures will, of course, be found in the central, older portion of the colony while the less developed ascocarps will be found to occur on the periphery.

The colonies established on agar plates from spores, mycelia, or fruiting structures taken directly from dung or other natural substrata will not necessarily be pure cultures, although they may well be unifungal and therefore suitable for a single laboratory exercise. If it is intended to use these cultures for subsequent laboratory periods, the fungi in question should be isolated and single-spore cultures established. Any of the several standard dilution plate methods recommended for single-cell isolation may be used, but because of the relatively rapid growth of these organisms, the individual germinating spores or germlings should be removed from the dilution plate as soon as germination can be detected, certainly within 12 hours.

Once a pure culture of any one of these organisms is established on an agar slant, it is relatively easy to maintain it in the pure state provided the initial incubation temperature is held at a high level. Any subsequent contamination will almost certainly be restricted to the thermotolerant *Aspergillus fumigatus* or to one or two thermoduric or thermophilic species of bacteria. If such contamination occurs, additional single-spore isolations may be necessary.

Established stock cultures of thermophilic fungi require very little attention. Since both the sexual and asexual spores produced by these organisms are long-lived, cultures may be maintained, even in a dry state, for periods up to several years without losing viability.

Laboratory Study

Aside from demonstrating peculiar temperature requirements, a few of the thermophilic fungi lend themselves well to general or specific laboratory studies. Their characteristic rapid growth, for example, makes them useful if for no other reason than that they can be worked into a laboratory schedule more easily than can some of the similar, slower growing mesophiles. In illustrating homothallism vs. heterothallism in the fungi, certain thermophiles, e.g., *Mucor pusillus*, *Myriococcum albomyces*, and *Thielavia thermophila*, could be used. For example, one of the two thermophilic species of *Mucor* (*M. miehei*) is homothallic and produces numerous zygospores over the entire surface of the agar in a few days at 40-50°C. The other species, *M. pusillus*, is heterothallic and invariably develops many zygospores, provided two compatible strains are crossed. Crossing is accomplished by placing the inocula of the two strains about 1" apart on the agar plate. The zygospores will not be widespread but will occur in a line in the center of the plate between the two colonies. These organisms are no more difficult to maintain and grow than are the mesophilic species usually used for such demonstrations and have the advantage of more rapid growth and great dependability.

Another thermophile that might well be incorporated into a general laboratory schedule is *Chaetomium thermophile* var. *coprophile*. It can either replace or be used as an adjunct to one or more of the mesophiles generally used for studying asci and ascospore development. This organism grows rapidly and unfailingly produces numerous perithecia containing easily viewed asci and ascospores. Because of its apparent widespread occurrence, it might very likely appear on collected natural substrata.

For illustrating extreme thermophilism in the fungi, there is no question that the rather common thermophile *Humicola lanuginosus* is the organism of choice. While its optimum growth occurs between 45-50°C, its upper temperature limit is considerably above the latter figure, some growth having been recorded at 59°C. It occurs rather commonly and may be expected to appear on natural material.

See also Emerson, 1941; Cooney and Emerson, 1964; Crisan, 1964; Fergus, 1964.

OSMOPHILIC FUNGI

In the discussion of yeasts (p. 124) there is to be found some reference to yeasts that grow well on media of high osmotic values. Certain species of *Aspergillus*, as well, have this capacity.

Aspergillus halophilicus is, without question, one of the most osmophilic of fungi. It will grow on substrates containing 50% sucrose but not at levels below this; it grows much better at a level of 70%. Salt (NaCl) can be used, but is less satisfactory than sucrose even at equivalent osmolarities. Glycerine, on the other hand, works very well and growth can be obtained at a level of 30%, which reflects, probably, the smaller size of this molecule. Despite the strict osmophily of this species, its growth rate is slow under the best conditions.

Members of the *Aspergillus glaucus* group uniformly grow much better on media containing 20% sucrose than at the much lower levels used for most fungi, and many of these grow much better when the sugar content is increased to 40%. Other species that give a striking response are: *A. arenarius* in the *A. versicolor* group and *A. penicilloides* in the *A. restrictus* group. The first of these on malt agar produces compact colonies that consist primarily of sclerotia, while it grows more rapidly and produces only conidial heads on malt-yeast agar containing 40% to 60% sugar. The second is strictly conidial and in three weeks produces colonies 5-6 cm in diameter on high sugar media as opposed to about 0.5 cm on malt agar at a sugar content of 4%.

Working with either *Aspergillus ruber* or *A. amstelodami* of the *A. glaucus* group, one can obtain colonies that show either abundant cleistothecia or none, depending on substrates and temperatures of incubation, although both are grown on osmophilic media. If grown at 25°C upon the conventional substrate (Czapek's solution agar with 20% sucrose), they form a dense layer of bright yellow cleistothecia with relatively few olive green conidial heads projecting above it. If, on the other hand, they are grown at 20°C on an agar medium containing 5% yeast extract, 3% NaCl, and 1% glucose, there are no perithecia whatever.

PART IV

FUNGI AS BIOLOGICAL TOOLS

21 Mechanisms of Spore Release and Dispersal

In this section are included a number of practical exercises on violent spore discharge, on nonviolent liberation, and on dispersal. The fungi selected for study represent a wide range of systematic types: Phycomycetes, Ascomycetes, Hymenomycetes, and Gasteromycetes. The organisms suggested for use can all, with a little experience, be used with confidence in experimental work. A substantial amount of additional information relating to light in relation to spore discharge is to be found at the beginning of Chapter 22, immediately following.

SORDARIA

Sordaria fimicola is a common coprophilous species that is easy to isolate and is stocked by most mycological laboratories. It is essential to use a culture in vigorous fruiting condition. Being homothallic, it produces perithecia over the whole surface of the medium; a discharging culture can be obtained in 10-15 days at 20°C and will remain active for at least 10 days; the distance of discharge is considerable (up to 10 or 15 cm). Since the necks of the perithecia are positively phototropic, the spore-guns can be accurately aligned by incident light.

In dispersal experiments with *Sordaria* and similar organisms it is convenient to grow cultures in small glass specimen tubes (2.5 x 2.5 cm). These can be dry sterilized, four at a time, in a deep (4-5 cm) Petri dish, filled to the brim with sterilized agar, and then inoculated (Figure 12).

For *Sordaria*, and for many other Ascomycetes, yeast-extract-filter-paper agar (M-61) is suitable.

Light and Spore Discharge

For short-period experiments on the effect of such external factors as light on spore discharge, an easily constructed box, conveniently made of black polymethyl methacrylate sheet ("perspex" or "lucite") as illustrated in Figure 13, can be used. For any one experiment at least two boxes must be available. A slide-holder, essentially a small lateral drawer, carries a microscope slide with a millimeter grid (1 cm^2) etched on its lower surface. When the slide-holder is pushed into position, the grid is exposed about 1 cm above the discharging perithecia. On removal, the number of spores shot onto the grid can be determined by counting under the microscope. To prevent undue drying it is best to include in the box a couple of specimen tubes containing water.

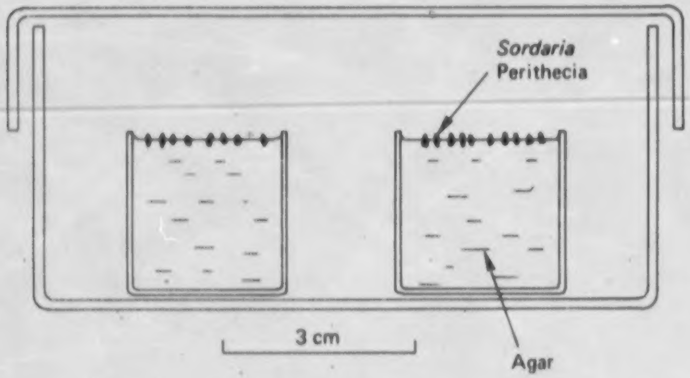

FIGURE 12 *Method of growing cultures for studies in Sordaria.*

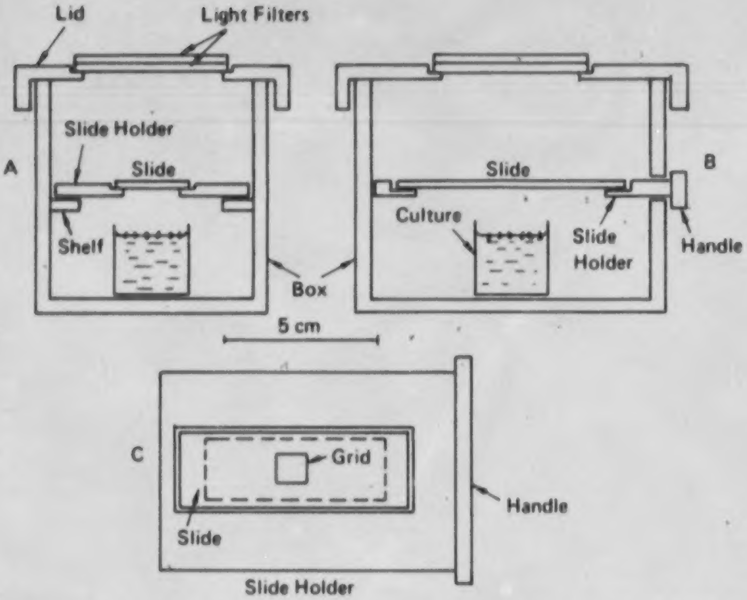
Lid
Light Filters
Slide Holder
A
Slide
Shelf
Box
Slide
B
Culture
Slide Holder
Handle
5 cm
C
Grid
Slide
Handle
Slide Holder

FIGURE 13 *Box used for the study of light and spore discharge.* A, *transverse section;* B, *longitudinal section;* C, *slide-holder in surface view.*

To study the effect of change from darkness to light, two sporulating cultures that have been raised in the light should be transferred one to each of two dark boxes and left blacked-out overnight. A small blob of plasticine on its bottom will fix the culture firmly in the box. The rate of discharge (conveniently determined as the number of spores discharged in half an hour) should be followed for both cultures the following day over a period of two to three hours. In one case the black shutter (an opaque square 5 cm^2) should be removed and replaced by a heat-absorbing clear glass filter of the same size and the culture illuminated at a known intensity, 50-100 ft-c from "daylight" fluorescent tubes being suitable. The second box remains as a "dark" control. For both cultures the half-hourly rate of discharge should be further followed for five to six hours. Graphs should be plotted, for each culture, of rate of discharge against time.

Many variants of the experiment can be tried. The effect of a single short light treatment (e.g., 5 min at 500-1000 ft-c) on subsequent discharge is very striking. Again the effectiveness of blue light in contrast to, say, yellow* is easily studied, but if the experiment is to be meaningful, the intensities of the two kinds of light must be equated using a selenium photoelectric cell, having regard to the transmission characteristics of the filters used and the variation of sensitivity of the selenium cell with wavelength (Figure 14).

Periodicity

For experiments lasting several days, a "spore clock" is a convenient type of apparatus and is easy to construct (Ingold and Marshall, 1963). It is (Figure 15) essentially a transparent "perspex" disk with, on its lower surface, a circular grid-band divided into small squares of about 1 mm^2 that rotates very slowly above a discharging culture. For some experiments a clock giving one rotation of the disk per day is suitable; for others a 7- or 8-day rotation may be preferable. By counting, under a microscope, the spores in each hourly section of the grid-band, data can be obtained relating rate of spore discharge to time. For counting, the disk must be removed from the spore clock and inverted

Various experiments can be carried out with this spore clock. It is particularly instructive to study discharge under a regime involving a 12:12-hour alternation of light and dark each day with temperature kept constant at, say, 20^{o}C. A light intensity of 20-100 ft-c from "daylight" fluorescent tubes is suitable.

It is also of interest to study the daily march of

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*Yellow is suggested rather than red, because at the red end of the spectrum the selenium cell is relatively insensitive. Light above 5400 Å does not appear to have any effect on *Sordaria*.

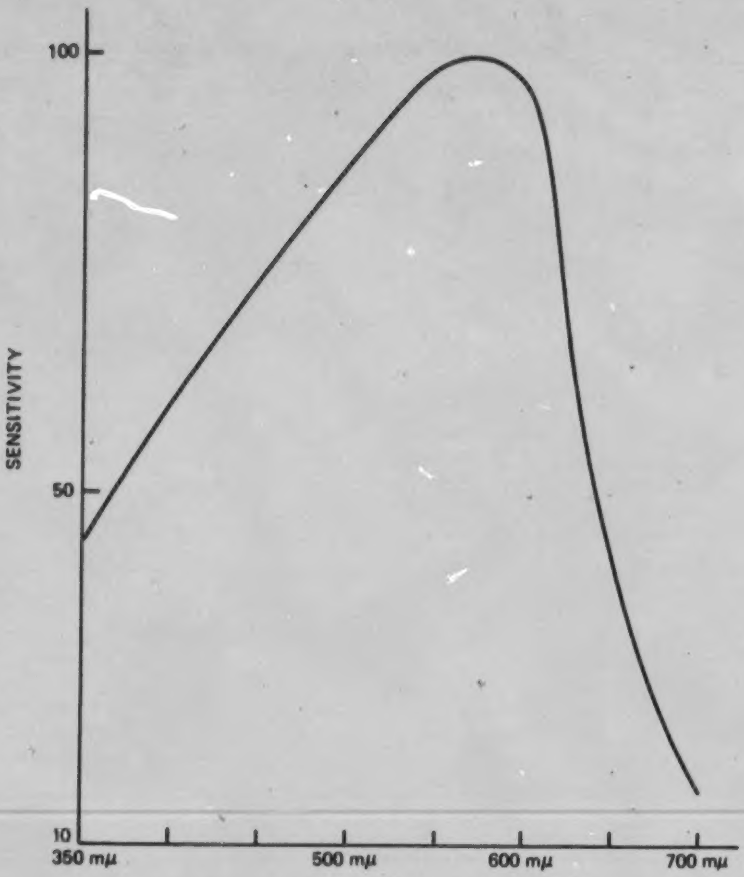


FIGURE 14 *Relative sensitivity of selenium photocell.*

rate of spore discharge in a culture exposed to the more natural alternation of conditions of light and temperature such, for instance, as may be encountered near a north-facing window. In such an experiment it is good to have a thermograph record of the daily temperature cycle and to record the period of natural illumination.

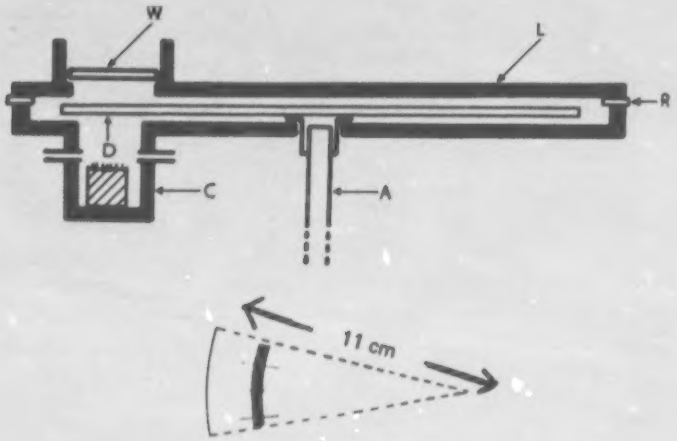


FIGURE 15 Above: *Sectional view of spore clock (1/3X).* L, *lid of box;* R, *rubber washer;* W, *light filter or opaque square;* C, *cubicle containing culture;* D, *rotating transparent disk;* A, *vertical rotating axis connected to clock.* Below: *A sector of the disk seen in surface view.*

Distance

The set-up recommended is shown in Figure 16. It consists of a long box with its interior walls having a black matte surface. A specimen-tube culture of *Sordaria* that has been grown under overhead lighting is placed horizontally on a wooden block shaped to receive it. It is illuminated by a horizontal parallel beam of light that passes through two holes 2.5 cm in diameter. These and the surface of the culture are carefully aligned. Any perithecia not already set horizontally should soon assume this position because of the positive phototropism of the necks. A day should be allowed for such adjustment. To maintain humidity satisfactory for discharge, small exposed water surfaces should be present in the inner section of the box.

With this arrangement, spores are shot almost horizontally and then fall onto a horizontal glass slide with a suitable grid etched on it. The trajectories of the discharged spores are presumably of the "sporabola" type, as discussed by Buller. After a few hours, when a light spore-deposit should have accumulated, the slide is removed and examined under the microscope. The spores on each transverse strip of the slide are counted to give data allowing the construction of a curve relating number of spores to distance of discharge. The complement of eight spores from an ascus may all stick together on discharge or may be broken up into single spores or groups of two to seven. It is of interest to plot curves of distance of discharge of single spores and of eight-spore groups. This can easily be done if the deposit is not too dense. In a dense deposit, scoring is made difficult by superimposition of projectiles.

A related fungus also interesting to use in this connection is *Podospora setosa*. Like *S. fimicola* it is a very reliable homothallic species. However, it requires longer (3-4 weeks) for cultures to reach a discharging condition. The ascus contains typically 256 spores, so

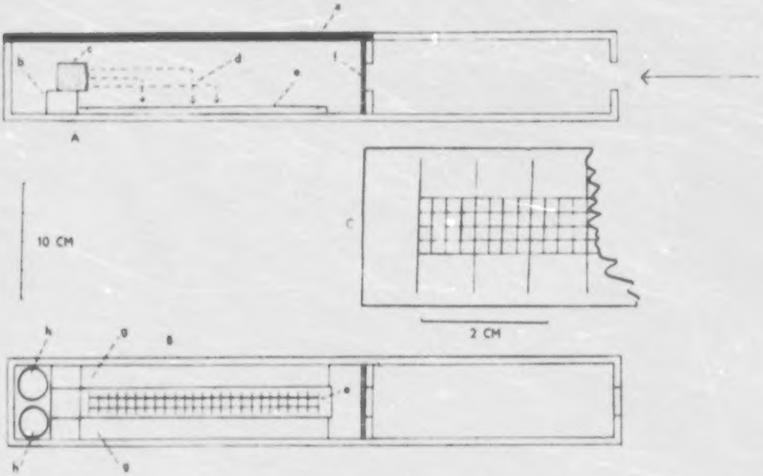


Fig. 16 Wooden box used in studying horizontal distances of spore discharge in *Sordaria*. A, vertical section of box: *a*, removable lid; *b*, culture holder; *c*, specimen-tube culture of fungus; *d*, trajectories of discharged spores; *e*, graduated slide; *f*, glass window. The arrow indicates the direction of the incident light. B, plan view of box; *e*, graduated slide; *g*, wooden guides holding slide in position; *h*, specimen tubes containing water. C, small part of graduated slide, at a larger scale, to show details of ruling.

that counting is rather difficult. Further, a very long discharge box must be used as spores may be shot to a distance of over 40 dm.

Entomophthora coronata (= *Conidiobolus villosus*) can also be used as experimental material. Cultures can be grown in specimen-tubes containing 2% malt agar and are ready to be used several days after inoculation. The conidia are discharged up to a distance of 4 cm. When a suitably dense spore deposit has accumulated, it is instructive to measure the diameter of, say, the five smallest spores in each transverse 1 cm-wide band of the slide. The results can then be compared with the theory that with a given vigor of discharge (as expressed by initial velocity) the distance (d) of discharge is given by $d = Kr^2$, where "K" is a constant and "r" the radius of the spore.

Analysis of Ascus Jets

This analysis can be made very simply using the arrangement shown in Figure 17. An electric motor has fixed to its spindle a horizontal transparent "perspex" disk 25 cm in diameter, rotating about 25 times per second just above the surface of a discharging culture of *Sordaria* near the edge of the disk. The ascus jets are caught in flight almost as soon as they leave the asci and are spread out horizontally. After a time the disk

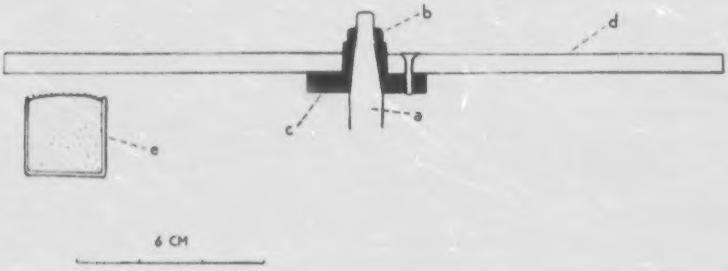


Fig. 17 Sectional view of apparatus used to study the form of ascus jets. *a*, spindle of electric motor; *b*, nut; *c*, brass socket made to fit the spindle; *d*, 'perspex' disk; *e*, fruiting culture of *Sordaria*.

can can be removed, inverted, and viewed under the microscope. Each impaled jet consists not only of spores variously grouped (e.g., {3-1-2-2} or {5-3} or {7-1}, etc.) but also of groups of granular ascus sap. It is instructive to make *camera lucida* drawings of a number of the different types of jet observed (see Ingold and Hadland, 1959).

SPHAEROBOLUS

Sphaerobolus stellatus is a beautiful and reliable organism for the study of violent discharge. It is easily maintained in culture without any loss of fruiting capacity, provided that each new culture is started from a discharged glebal-mass. The use of mycelial inocula soon leads to reduced fruit body production and finally cultures become completely sterile.

The fungus will fruit on a number of common laboratory media, but oat agar (M-15) is much the most satisfactory. Since cultures take several weeks before being ready for use in an experiment, they should be on a relatively deep layer of agar, and precautions should be taken to prevent undue desiccation.

Fruiting is absolutely light-dependent. A good standard procedure is to inoculate Petri dish cultures each with a central glebal-mass and leave in darkness for three weeks. The cultures are then brought into continuous light of 50-100 ft-c from "daylight" fluorescent lamps. Cultures may be raised from the outset in light but more uniform fruiting seems to result after initial growth in darkness. Since the tiny spherical sporophores are phototropic, illumination should be from directly overhead.

Temperature control is important. At a temperature of 18-20°C discharging fruit bodies are formed in 10-15 days after bringing a culture into light. However, above 25°C fruiting is completely inhibited in the strain in use in Ingold's laboratory. Probably, since the fungus has a world-wide distribution, the temperature at which inhibition of fruiting occurs differs with isolates from different regions.

Once fruiting starts, and provided there is enough agar and the culture is not allowed to dry out, glebal-mass discharge in continuous light goes on for months, but with an innate periodicity having about 12 days between successive peaks at 20°C.

Alternation of Light and Darkness

If manual operation of the light control is involved, a regime of 16 hours darkness and 8 hours light each day (rather than 12:12) is suggested. Discharge can be assessed by the number of glebal masses shot onto the lid of each dish. A group of 4-8 dishes should be adequate and it is convenient to use cultures in which discharge has just started. For each light and each dark period the average hourly rate of glebal-mass discharge can be determined and a suitable graph plotted over any desired period (e.g., 10 days).

Another parallel group of plates can be used to study a regime involving 24 hours light and 24 hours darkness.

Under the first regime glebal masses are discharged in the light periods; in the second, in dark periods. It is a useful exercise for the student to speculate on the reason, still unknown, for this difference. Clearly, other regimes can be used and it is of interest to follow the course of discharge over a period of a week in continuous darkness following any one type of light:dark treatment.

Distance

It is best to use fruiting cultures grown in small Petri dishes (5.5 cm) in which a number of sporophores have already split in a stellate manner, exposing their glebal masses. Such fruit bodies will normally discharge their glebal masses within four hours. Take a deep beaker or jar, with a diameter significantly greater than the small Petri dish, and line the sides of this with wet filter paper. Remove the lid of a suitable culture and fix it to the bottom of the jar with a blob of plasticine. The object of the jar with its lining of filter paper is to maintain a damp atmosphere around the discharging culture. The whole set-up (Figure 18) can be regarded as a wide-barrel gun and can be tilted at an angle of about 45°C to the vertical. A roll of white paper can be unrolled horizontally from the gun along its line of fire to a distance of up to 6 m. The whole can be left for several hours and then the discharged glebal masses can be identified on the paper and their distance of horizontal discharge recorded. If the roll of paper used is about a meter wide, it is convenient to use a battery of parallel "*Sphaerobolus* guns" side by side.

PATTERN OF AERIAL DISPERSAL

This experiment illustrates the important idea of the rapid attenuation of spore deposition around a center of spore liberation. Essentially, very large numbers of spores must be liberated at a center and trapped at increasing distance.

A fully mature ostiolate puff-ball (*Lycoperdon* sp.) or earth-star (*Geastrum* sp.) is a very convenient source of spores. A supply of sporophores can be collected in the proper season and stored without squashing for future use. In these fungi spores are mostly set free by falling raindrops. A large drop striking the paper-thin, unwettable peridium momentarily depresses it and causes a puff of spores to be ejected through the ostiole to a height of 10-20 cm. If this is done in front of a projector, so that a shadow falls on the screen, it is an effective lecture demonstration.

In the present experiment a dripping system can be arranged that allows a drop to strike the peridium (but not the ostiole) about once a minute (Figure 19).

Along a number of radii from this puffing center, horizontal slides are placed on the ground at intervals extending to perhaps 20 m. Each slide should have etched on it a square centimeter divided into mm^2. It may be best for the surface to be made sticky by a very thin smear of vaseline. Using suitable meteorological instruments, the wind direction and velocity are recorded at the height of the spore puffs.

After about a half-hour, the dripping mechanism

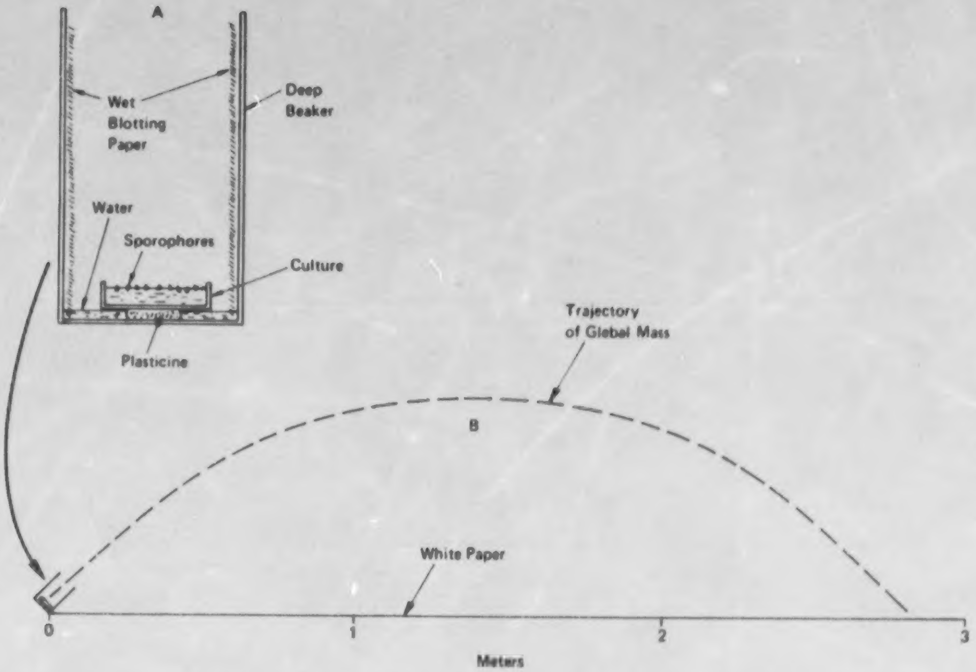

A
Wet Blotting Paper
Deep Beaker
Water
Sporophores
Culture
Plasticine
Trajectory of Global Mass
B
White Paper
0
1
2
3
Meters

FIGURE 18 A. *Arrangement for holding discharging cultures;* B. *Set-up to determine distance of throw.*

generating the puffs of spores should be stopped and an additional half-hour left for any residual spores to settle or be dispersed from the experimental area. The slides are then collected, searched under the microscope, and the number of spores deposited on 1 cm^2 determined.

For each radius, a graph is constructed of spores deposited per unit area plotted against distance from source. A plan diagram can also be made of lines of equal spore deposition around the center and indicating mean wind direction.

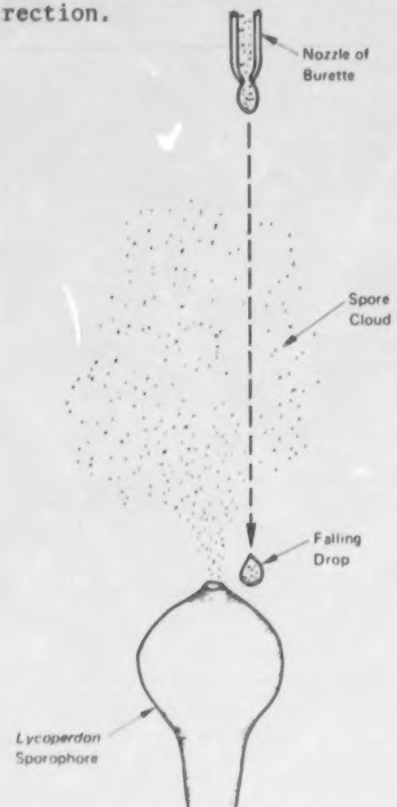

Nozzle of Burette
Spore Cloud
Falling Drop
Lycoperdon Sporophore

FIGURE 19 *Arrangement for study of dispersal around a center.*

MUCORS

Speed of Realignment of Pilobolus

An interesting experiment with *Pilobolus* is to study the speed with which the phototropic spore-guns, aligned by light from one direction, can be realigned by illumination from a new direction. Under natural conditions of lighting, discharge is strictly periodic, beginning in the late forenoon and ending by early afternoon.

The arrangement shown in Figure 20 is suitable. An approximately cubical cardboard box (about 20-25 cm) is painted black inside to give a nonreflective surface. Two small square holes 2-3 cm across are cut in the lid, as far apart as possible, on either side of the central point, and covered on the inside by small glass sheets each about 10 cm^2. These are secured in position by adhesive tape. Thus two small windows are provided, either of which can be obscured at will. A duplicate lid similarly equipped should also be prepared.

A few dung balls that have been under natural conditions of illumination, with good crops of yellow developing sporangiophores, are placed in the evening in the box together with a few beakers of water to maintain humidity. Early next morning the sporangiophores are illuminated through one of the windows, the other remaining blacked out. Discharge should start around 10-11 am. When perhaps 20-50 of discharged sporangia have accumulated on the glass of the exposed window, the lid of the box should be removed, replaced by the duplicate one and now illuminated from the second direction.

Examination of the glass plates from the first lid should indicate that sporangia are to be found only on the plate through which illumination had occurred. During the next few hours the newly-illuminated window should be kept under observation. When discharged sporangia appear on it, there is clear evidence that the spore guns have become realigned. In this way the time necessary for realignment can be determined.

Distance of Discharge in Pilobolus

A set-up essentially similar to that proposed for *Sphaerobolus* can be used. It is interesting to compare the respective distances in these two fungi in relation to the sizes of their projectiles.

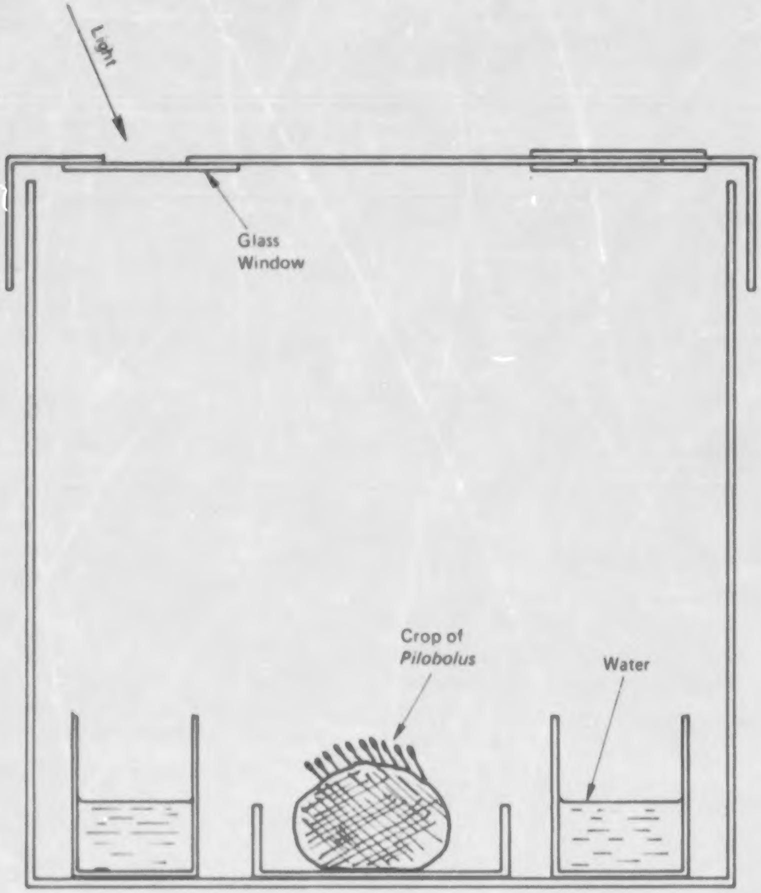

Light
Glass Window
Crop of *Pilobolus*
Water

FIGURE 20 *Arrangement for studying readjustment of Pilobolus to light.*

Liberation of Spores from Mucor

This simple experiment demonstrates how different, from the point of view of spore liberation, may be the behavior of the same morphological type of spore-bearing structure.

Suitable species to use are *Mucor ramannianus*, *M. hiemalis*, *M. plumbeus* (= *M. spinosus*) and *M. petrinsularis*. Cultures are grown on 2% malt agar in deep (5 cm) Petri dishes or in suitable beakers covered with Petri dish lids. When a culture is sporulating freely, the lid is removed and it is inverted over a microscope slide. The base of the dish, now uppermost, is then tapped vigorously. After a short interval, to allow any liberated spores to settle, the slide is examined for spores and for fragments of sporangium wall.

Under these conditions, and also when suitably subjected to blasts of air, no spores are set free from *M. ramannianus* and *M. hiemalis*, but spores, usually in groups, are freely liberated from the other two species.

SPLASH LIBERATION

There is a growing realization of the importance of rain-splash in connection with spore liberation, particularly when "slime-spores" are involved. The following experiment is based on the fundamental studies of the problem by Gregory *et al.* (1959).

Drops of water 3-5 mm in diameter are allowed to fall from a height of 2-3 m onto a horizontal fungus target, the reflected drops are then collected at increasing distances and their spore content noted. The experiment should be conducted indoors under still conditions so that the path of the splash drops is not affected by movements of the air.

It is suggested that the target should be a 2-cm disk cut from a sporulating Petri-dish culture and placed on a glass slide. *Fusarium fructigena* and *Mucor ramannianus* (a form with very short sporangiophores) serve well in this connection. If a more natural target is desired, a suitable portion of bark bearing the conidial stromata of *Nectria cinnabarina* is very appropriate.

Reflected droplets are caught on horizontal glass slides placed at increasing distances (up to 50 cm) from the target. The principal technical problem is to recognize the droplets, which dry up very quickly. This is done by using slides coated with a thin gelatine film, stained with Naphthol Green B.

In the preparation of these, 1 g of the stain is dissolved in 20 ml warm distilled water, filtered, and added to 10 ml of a 4% (W/V) solution of gelatine. This solution is used to coat the slides. Microscope slides are washed in chromic acid or suitable detergent and dipped in ethanol. They are then polished with a clean, grease-free cloth. Two drops of the green solution are then placed on a slide and spread over the whole surface, using the edge of another clean slide. The slides are allowed to dry and should then be fairly evenly-stained green.

A droplet falling on a green trapping-slide and then drying leaves a clearish disk with an intensely green circumference, and the diameter of this may be taken as approximately three times that of the droplet producing it (Gregory *et al.*, 1959). For each drop hitting the target area the number of reflected droplets caught on the slides can be counted and their size distribution can be noted. Further, by microscopic examination, the number of droplets with spores can be determined. It may be a little difficult to see the spores in the dry state and the addition of water causes immediate solution of the dye and the destruction of the splash areas. However, if drops of liquid paraffin are placed on the slide and covered with a cover slip, observation under the high power of the microscope is easy and the splash areas retain their identity. Most of the spores are to be found in the deeply-stained circumference forming the outer limit of the splash drop. Of course, only a small part of the total splash area is sampled, and multiplication by the appropriate factor is necessary to get figures for the total number of droplets produced from a single falling drop.

Curves can be constructed of number of droplets plotted against distance, and the number of spore-bearing droplets plotted against distance. With this simple technique, many variations in design of the experiment can obviously be used and many different kinds of targets can be tested.

See also Ingold and Dring, 1957; Gregory, 1961; Alasoadura, 1963.

22 Fungus Physiology

RESPONSE TO LIGHT

Visible light influences fungi by affecting the rate of systhesis of compounds, by entraining circadian rhythms, by inducing the formation of specific structures, or by influencing the rate or direction of growth.

Tropisms

Pilobolus The species of *Pilobolus* fall into three groups that may be recognized by the following characteristics: (1) *Pilobolus kleinii* and similar species grow rapidly, so that the mycelium fills a 100-mm Petri dish in five days on asparagine-acetate medium (M-78); it forms no trophocysts in darkness; the sporangial wall usually darkens before the subsporangial swelling forms and the spores are ellipsoid and orange-yellow in color; (2) *P. sphaerosporus* has similar characteristics, except that it is capable of forming trophocysts and sporangia in darkness, and the orange-yellow spores are spherical; and (3) *P. crystallinus* and its relatives have mycelia whose growth tends to be restricted on asparagine-acetate medium; they form trophocysts but no sporangia in darkness; the subsporangial swelling enlarges before the sporangium darkens, and the ellipsoid spores are only very pale yellow in color.

When darkness is called for in an experiment, complete darkness must be provided, because the fungus is very sensitive to light. Even the light from a Bunsen burner suffices to induce trophocysts in *P. kleinii*, for example, and young sporangiophores show a phototropic response to very low intensities. Sporangiophores of *Pilobolus* are capable of phototropic response at two stages of their development: (1) Young sporangiophores that have emerged from the medium but whose tips have not begun to swell respond to unilateral illumination by bending or by growing toward a source of light; and (2) mature sporangiophores bend toward a light.

To observe the response of young sporangiophores, *P. kleinii* is grown in 60-mm Petri dishes containing about 8 ml of ammonium sulfate-acetate medium (M-75). Cultures are inoculated with hyphal tips and incubated in the dark for 4 days. They are then exposed to light for 12 hours, returned to darkness for 12 hours, and again exposed to light. The intensity of the light is not critical; about 5 ft-c from fluorescent tubes is satisfactory. Young sporangiophores will have emerged from the medium by 12 hours after the beginning of the second light period, and they are responsive to unilateral illumination for 2 hours or more.

Qualitative demonstrations of phototropism may be prepared by directing a small microscope illuminator on a culture or by arranging a desk lamp in such a way that the sporangiophores are illuminated from one side. The response is evident within 1 hour after the beginning of exposure, and after 2 hours it is striking when viewed with a pocket magnifier or a dissecting microscope.

Young sporangiophores are suitable for laboratory demonstrations and exercises because they can be produced in quantity in accordance with a time schedule and show a clear response within a single laboratory period. They also lend themselves well to many types of experiments that can be performed with simple, inexpensive equipment, i.e., the effect of simultaneous exposure to two light sources, tests of spectral limits, or tests of the effects of probing the sporangiophore with narrow beams of light.

The technique for growing mature sporangiophores is very similar to that used for producing young ones. Cultures of *P. kleinii* are grown in 60-mm Petri dishes of ammonium sulfate-acetate medium at 25°C in darkness for 4 days. They are then subjected to a daily alternation of 12 hours of light and 12 hours of darkness. Sporangia are discharged approximately 3 hours after the beginning of the third light period, and sporangiophores are phototropically responsive for several hours before the time of discharge. The beginning of experiments on phototropism can be scheduled for 12 hours after the end of the second light period. Mature sporangiophores show a clear phototropic response 1 hour after the beginning of unilateral illumination. The response may be observed with a pocket magnifier or dissecting microscope, or it may be deduced later from the pattern of distribution of discharged sporangia adhering to a target.

Spectral limits can be demonstrated by exposing cultures to lights of various wavelengths or by projecting a spectrum on a culture and interposing a sheet of glass between the culture and the prism. Other types of experiments will be obvious to a student with an attitude of enquiry, and one of the few precautions that must be observed in arranging them is that the relative humidity of the atmosphere surrounding the sporangiophores must be maintained at a high level at all times.

Entomophthora Various saprophytic members of the Entomophthorales discharge their conidia toward a source of light, and this response can be made the basis for laboratory exercises and demonstrations on phototropism. They perform reliably and are easy to isolate, maintain, and manipulate.

Cultures of *Entomophthora coronata* or species of *Conidiobolus* suitable for experiments with light may be obtained from various culture collections or they may be isolated from leaf mold by the "canopy plate" technique. Leaf detritus is an excellent source of saprophytic Entomophthorales. Decaying leaves of either coniferous or broad leaved species are satisfactory, and best results have been obtained with material collected close to the soil beneath a fairly thick layer of fallen leaves. The detritus can be transported to the laboratory in a plastic bag or other convenient container. Canopy plates are prepared by using tapwater agar to cement leaf detritus to the underside of the lids of Petri dishes containing nutrient media. Conidia shot from conidiophores on the detritus germinate and give rise to visible mycelia within three to seven days. Pure cultures may be established by placing small blocks of medium cut from an active colony on the underside of the lids of Petri dishes containing fresh sterile medium. Cultures grow well at 22-25°C on potato glucose agar (M-19) or asparagine-glucose (M-81) medium.

The response of *Entomophthora coronata* to unilateral illumination makes a simple yet striking demonstration of phototropism. If a Petri dish is inoculated with conidia at a single point and exposed to light from one side, a swath of discharged conidia and young mycelia extending from the point of inoculation toward the light will be clearly evident within 4 days at 25°C. Although this demonstration cannot be carried out within a single laboratory period, it is clear-cut, reliable, and requires a minimum of equipment.

Entomophthora coronata can be used to advantage for many other types of classroom demonstrations or individual projects. For example, experiments dealing with the effects of intensity or of intermittent illumination might be instructive, and clear results can be obtained from tests of the response to simultaneous illumination with two light sources that differ in intensity or in the angle that they subtend with respect to the fungus. For some of these investigations, 7.5-watt frosted incandescent lamps in small porcelain sockets make convenient point sources of light, and the fungus responds well if the lamps are located 5°-10° above the plane of the surface of the medium. With more elaborate equipment, the spectral limits for the phototropic response may be investigated either by means of filters or by means of a spectrum projected on a culture. Colored lights may also be used, but students should be cautioned that most of them are by no means monochromatic.

Other species: *Aspergillus giganteus*, *A. clavatus*, *Sporodinia grandis*, and *Phycomyces* may be handled in much the same way as *Pilobolus* and *Entomophthora*.

Entrainment of Circadian Rhythm

Since it is capable of producing all parts of its asexual reproductive apparatus in darkness, *Pilobolus sphaerosporus* is probably the best suited species of the genus to experiment on rhythms. Other species will discharge their sporangia periodically in response to periodic alternations of light and darkness. *P. sphaerosporus* may be grown on either dung extract agar or asparagine-acetate medium, and either asparagine-acetate medium or ammonium sulfate-acetate medium is suitable for use with *P. kleinii*. Mycelia growing in Petri dishes can be subjected to various programs of illumination (a daily alternation of 12 hours of darkness will induce periodic sporangium discharge in most strains), and the discharge of sporangia may be followed by collecting them on the lids of Petri dishes that are changed periodically, or microscope slides that are replaced at intervals. More elaborate equipment can be devised to collect the discharged sporangia on moving cellophane tape or on a plastic disc that is rotated over a culture. Improvization of adequate and reliable apparatus should challenge an ingenious student.

Induction of trophocysts in *P. kleinii* is a simple and reliable demonstration of a morphogenetic response of a fungus to light. This species forms no trophocysts if grown in darkness, but numerous trophocysts are formed by a mycelium after only a brief exposure to blue light (390-510 mμ).

One procedure for demonstrating the effect of light on trophocyst formation is as follows: A suitable number of 60-mm Petri dishes (either glass or plastic) of ammonium sulfate-acetate medium (M-75) or asparagine acetate medium is prepared and inoculated with hyphal tips cut from the periphery of an active mycelium. If it is desired to eliminate all trophocysts from control plates, cultures must be kept in darkness for several passages, and inoculation must be performed in a darkroom under a red photographic safelight with the cultures shielded from the light of the Bunsen burner. If a few trophocysts near the point of inoculation can be tolerated, inoculation can be carried out in light. After inoculation, the plates may be wrapped carefully with aluminum foil or they may be placed in a light-tight box and incubated in darkness for 4 days at 25°C. On the fourth day, all of the cultures except the controls are exposed to light (an exposure of 100 sec to 50 ft-c from fluorescent tubes is sufficient). The plates are then returned to darkness. After 24 hours, the mycelia that were exposed to light should have formed numerous trophocysts, while the control mycelia should have formed none, or only a few. Variations and extensions of this general procedure are obvious, and the system is well suited to experiments concerning the dose of light, spectral limits, or even action spectra.

See also Berliner, 1961a; Sussman *et al.*, 1964; Sargent *et al.*, 1966; Bourret *et al.*, 1969.

Spore Discharge (See also Chapter 21)

Pilobolus For accurate timing, cultures grown on synthetic media and synchronized with a program of illumination are most satisfactory. Moreover, on transparent agar media, all parts of the asexual reproductive apparatus, including the trophocysts and young sporangiophores, may be observed easily with a dissecting microscope. Since the precise moment of discharge is influenced by temperature, not all sporangia are discharged simultaneously, and strains may differ somewhat in their behavior, it is probably wise to schedule the performance for the middle of a laboratory period.

Perhaps the simplest demonstration is merely to distribute Petri dish cultures to students. Each student can then observe the reproductive structures and discharged sporangia with a dissecting microscope. If the cultures are properly timed, and if there is an abundant crop of sporangiophores, most of the students will catch a glimpse of a discharging sporangium, and although the motion is too rapid for the eye to follow, it is still exciting.

If a dish is held with the lid against the ear, the "plink" made by a sporangium as it strikes the lid may be heard distinctly. The sound may be amplified easily to make it audible to an entire class if before the anticipated time of sporangium discharge, the lid of a Petri dish is removed, and a piece of thin polyethylene or other plastic food wrapping material is stretched tightly over the bottom half of the dish and held in place by a rubber band. The culture is placed under a light and a crystal phonograph cartridge so arranged that a short piece of wire inserted in the needle chuck is in contact with the taut plastic film. The phonograph cartridge is connected to an audio amplifier and loudspeaker (the inexpensive imported phonograph cartridges, transistor amplifiers, and loudspeakers sold by mail-order electronics supply houses are satisfactory).

The accuracy with which sporangia are directed toward an illuminated target may be demonstrated as shown in Chapter 21.

Other Species Discharge of sporangia of *Conidiobolus villosus* and ascospores of *Sordaria fimicola* toward light may be demonstrated by wrapping Petri dish cultures with aluminum foil and cutting holes, slits or designs through the foil on the lid. Cultures are then incubated beneath a light source and the spore deposits observed after a few days, comparing them to cultures that have remained in complete darkness.

Sphaerobolus stellatus may be used to demonstrate the effects of periods of alternate light and darkness on discharge of the glebal balls. It is best to use cultures in which discharge has just started. Natural light from a north window should be satisfactory if facilities for using artificial light are not available. The relative rate of discharge during the light and dark periods can be determined by counting the number of glebal balls stuck to the lids of the dishes. Under these conditions discharge occurs mostly during the light periods.

Production of Fruit Bodies or Spores

Increased Spore Production In this exercise, use *Trichoderma lignorum*, which produces green spores in light. Use yeast extract glucose agar (M-64) and inoculate the plates at the center with a few spores.

Place at least nine plates in continuous darkness, three under alternating light and darkness (12 hours each), and six under continuous light. At the end of 2 days, or when cultures have reached a diameter of about 2", remove six plates from darkness and place in continuous light. Return three of these plates to darkness after 12 hours' exposure to light and at the same time remove three plates from the continuous light and place in darkness. When the cultures are 4-5 days old, observe sporulation on all plates. Abundant green spores are produced only on mycelium that was young when exposed to light. Relatively few spores are produced in darkness and these are not so highly pigmented. The cultures under continuous light should show uniform abundant sporulation.

Production of Fruit Bodies or Spores *Schizophyllum commune*, *Dendrophoma obscurans*, or *Epicoccum nigrum* are suitable test species. Suggested media are casamino acid glucose agar (M-34) or yeast extract glucose agar (M-64). Inoculate several agar plates with the selected species and place them under three conditions: Continuous darkness, alternating 12 hours light and 12 hours darkness, and continuous light of 70-100 ft-c. Incubate 10-14 days near 25°C. At the end of 4-6 days remove part of cultures of *E. nigrum* from darkness, expose for 24 hours in light and return to darkness. Under these conditions the requirement for light should be evident.

To demonstrate that certain wave lengths of visible light are more effective than others, wrap the Petri dish cultures in different colors of cellophane or use cellophane as filters between the light source and the cultures. If more sophisticated equipment is available, one may set up a series of monochromatic filters and determine more accurately which wave lengths are more effective. Most of the fungi tested respond to the shorter portion of the visible spectrum.

If one wishes to use slime molds to illustrate the light requirement for fruiting, four species are suggested: *Physarum cinereum*, *P. nicaraguense*, with white plasmodia, and *P. flavicomum* and *P. tenerum*, with yellow plasmodia. Follow techniques outlined in Chapter 5 for media and other conditions necessary for production of sizeable plasmodia in darkness. Transfer blocks of agar bearing plasmodium to plates of cornmeal agar and return some to darkness. Incubate as before. When cultures in light have fruited, examine those in darkness and compare the results.

Intensity and Duration *Epicoccum nigrum* is a good test fungus to demonstrate two additional characteristics of certain fungi. This species requires a higher intensity of continuous white light (above 30 ft-c) than do most fungi. The length of exposure required for sporulation varies inversely with the intensity. Place inoculated agar plates in darkness for 4-5 days and then transfer them to following conditions: Continuous light of 5-10 ft-c; continuous light of 100-200 ft-c; 30 min of light at 100-200 ft-c; 8 hours at 100-200 ft-c; 30 min at 2,000-5,000 ft-c (direct sunlight through lids of dishes). Return all plates except those under continuous light to darkness and allow 4 days for development of spores. A short exposure at high intensity light should be as effective as long exposure at low light intensity for sporulation of this fungus.

Replacement by Components of Natural Medium *Dendrophoma obscurans* is suggested as a test species. Here, in addition to the casamino acid glucose or yeast glucose medium given above, collect a few green strawberry leaves, place the leaflets in Petri dishes on moist filter paper and autoclave.

Inoculate both the agar medium and the autoclaved leaves with pieces of mycelium and place some cultures under continuous light, alternating light and darkness, and continuous darkness. A third medium may combine the artificial medium and autoclaved leaves. After 14-21 days, remove cultures from darkness and observe sporulation on the strawberry leaves. The precise nature of the nutrient in the leaves that replaces the light requirement on agar medium is not known.

Sporulation in Light and Dark Alternating periods of light and dark (about 12 hours each), or a period of growth for 2-3 days in continuous light followed by several hours in darkness, are necessary for the production of sporangia of *Sporodinia grandis* and for the conidia (sporangioles) of *Choanephora cucurbitarum*. A yeast extract glucose agar (M-63b) is suggested. Use spores or young mycelium as inoculum. Inoculate several agar plates and place under following conditions: Continuous darkness; darkness 2 days, light 1 day; continuous light 50-100 ft-c; 2 days continuous light, 1 day darkness; alternating 12 hours darkness, 12 hours light. (After 2 days, colonies should be about 2" in diameter; if less, wait another day before changing cultures to the second condition.) Caution should be taken to use only Petri dishes with loose-fitting lids or to raise the lids slightly with thin metal strips to provide better aeration for *C. cucurbitarum*. These two fungi die out quickly in culture and can be maintained best as dry spores, but fresh cultures should be started every month.

Production of Sporangia and Oospores *Phytophthora cactorum* (homothallic) should be grown under these conditions: Continuous darkness; alternating 12 hours light, 12 hours darkness; continuous light of 5-100 ft-c. Use either lima bean agar (M-14) or glucose asparagine agar (M-79) with a few crystals of beta-sitosterol sprinkled on the surface. Inoculate cultures near 20°C for 5-8 days. Oogonia with paragynous antheridia are usually abundant in cultures grown in darkness, the greater number being formed in continuous darkness. Sporangia are more abundant in cultures receiving light and are more often formed on the surface of the agar. Mature oospores should be formed quickly under these conditions.

Fruiting of Cyathus stercoreus Doubtless other Basidiomycetes would be useful for this purpose, but the diploid mycelium of *Cyathus stercoreus* performs admirably (Brodie, 1948, 1949). Not all strains fruit equally well and the fruiting capacity should be determined beforehand.

A number of agar plates (M-53) should be inoculated simultaneously from the stock culture. Wrap some of these (perhaps 8 out of 16) in colorless cellophane, four (each separately) in red cellophane (usually two thicknesses), and four more in blue cellophane. Place all plates for 10-14 days in the dark at the same temperature as will be used later for exposure to light. All plates, except the four to be kept in the dark, are then placed about 2 feet beneath two 40-watt cool-white fluorescent bulbs.

The cultures should be allowed to grow until fruiting bodies are fairly well developed in the plates that are covered only with colorless cellophane; this requires 30-45 days, depending upon the strain of the fungus. The four sets of plates are compared after being labeled. Results are usually as follows: Total darkness, no fruiting; white light, abundant fruiting; blue light, abundant fruiting; red light, no fruiting.

The experiment may be refined by using filters instead of cellophane and may be varied by using a range of colors, by covering one half of a plate with red and one half with blue, etc.

Pigment Production

Plasmodial Pigments of Slime Molds Start about six cultures of each of the following species by plating spores on cornmeal agar: *Physarum flavicomum*, *P. gyrosum*, and *Didymium squamulosum*. Incubate three cultures of each in light and three in darkness. When plasmodia develop, feed all cultures with pulverized sterile oat flakes. When plasmodia have covered the surface of the agar, compare the color of those in light with those in darkness.

Carotenoid Pigments in Dacryopinax spathularia This species is suggested because it produces many separate cells or small clumps of cells in liquid media. A yeast extract glucose liquid medium (M-62) and Erlenmeyer flasks or test tubes can be used. Inoculate several flasks of medium and incubate near 25°C. Place some of the cultures under white fluorescent lamps (100-200 ft-c) and the remainder in continuous darkness. After 20-25 days compare the color of the cultures, which should show bright yellow pigmentation of cells grown in light but little pigmentation of cells developed in darkness.

This protective function of the yellow carotenoid pigments against strong sunlight can be demonstrated by comparing the rate of photooxidative killing of the light-grown cells with that of the dark-grown cells. Shake the liquid cultures until many separate cells are present, allow the clumps to settle out, and transfer the upper cell suspension to two sterile tubes, one with dark-grown cells and the other with light-grown cells. If the numbers of cells are very unequal, dilute one suspension until both tubes contain approximately the same number per unit volume. Expose the tubes or flasks of cell suspension to bright sunlight of 2,000-10,000 ft-c for 2 hours. As a control, paint some of the tubes black or wrap in black paper to shield from the direct rays of the sun. After exposure, transfer 1 ml of cell suspension to each of several agar plates and flood the surface evenly. Allow a few days for the cultures to develop in darkness and count the number of colonies produced by exposed dark-grown cells, exposed light-grown cells, unexposed dark-grown cells, and unexposed light-grown cells. The relative number of viable cells under each condition can then be compared.

Conidial Size

For those who have permanent equipment for light studies, and recognizing the variability of the medium--*Helminthosporium* spores are known in at least one species to vary with sugar concentration--it is still possible to show substantial effects of light on conidial size. Cultures of *H. rostratum* grown on V-8 agar (M-29, 30) in an incubator under fairly bright light have conidia about twice as long as those grown beside them but kept in the dark by wrapping the plates in metal foil. A series of intensities and sizes can also be used, but is substantially more complicated to set up.

LUMINESCENT FUNGI

All the well-studied light-emitting fungi are Basidiomycetes and are restricted to the Polyporaceae and

Agaricaceae. The best known and most easily obtainable are: *Armillaria mellea*, *A. fusipes*, *Pleurotus ostreatus*, *Clitocybe illudens*, *Panus stipticus*, *Mycena polygramma*, *M. galopus*, *Omphalia flavida*.

Depending upon the species, the fruiting body, in whole or in part, or only the mycelium or both, may be luminescent. Light emission can be demonstrated by either the fruiting body in a moist chamber or the mycelium grown in culture. *Armillaria mellea*, *Panus stipticus* or *Clitocybe illudens* are the most reliable producers of light for classroom demonstrations.

Armillaria mellea

Armillaria mellea, the honey agaric, is found on old stumps, dead trees, or buried roots. It is widely distributed throughout the temperate regions of the world, particularly on dead or dying oak or birch trees. The mycelium, rather than the fruiting body, is luminous. Mycelia, both in nature and in culture, are characterized by rhizomorphs or hardened masses of mycelial strands. These are whitish at first but must have aged to the point of being brown before they will show noticeable luminescence. As the rhizomorphs get still older, turn dark, and are covered with a cuticle, the luminescence disappears. With old age drops of dark brown, nonluminous fluid are exuded.

To maintain the mycelium in culture and to obtain maximum sustained luminescence, bread-crumb agar (M-2) is most satisfactory. The cultures can also be maintained on Sabouraud's glucose agar, but luminescence will be weak. Bread-crumb agar is also the most satisfactory medium for *Panus stipticus* and *Clitocybe illudens*. It is best to maintain stocks and to make demonstration cultures on agar slanted in 250-ml Erlenmeyer flasks, since it takes a minimum of two weeks for light output to be noticeable. Subculturing for stock maintenance need be done only three or four times a year.

In cultures at 21-25°C, maximum luminescence will occur after 3 weeks and may last as long as 10 weeks. It must be remembered that the greenish light can be seen only in a completely darkened room and that it takes the average individual at least 10 min to become sufficiently dark-adapted to discern these low light levels. Accurate measurements of light intensity levels for luminescent fungi in cultures can be made only by using a photomultiplier tube and recording accessories.

The luminescence of *A. mellea* can be demonstrated in nature by examining white mycelium-containing rotted wood. If it glows in the dark, it is most likely to be *A. mellea*. It can also be recognized by the black shoestring-like rhizomorphs. If the pieces of wood are kept damp in a moist chamber, they can provide very good laboratory evidence of luminescence for several weeks, but are suitable only for crude physiological experimentation, i.e., demonstration of necessity of oxygen for light output.

Cultures can also be obtained by germinating basidiospores on bread-crumb agar.. Mycelium arising from single spores will not luminesce for long, and dikaryotization must be allowed to occur. Thus, no attempts at single spore isolates are necessary.

Panus stipticus

Panus stipticus is a luminescent wood-destroying fungus whose fruit bodies are found in clusters on the dead stumps or logs of oaks, birches, alders and other Temperate Zone trees. After several weeks or even months, dried material can be soaked in water and within an hour the pileus will begin to swell and will become fully extended within 2-5 hours. At the same time, the extremely small basidiospores are discharged and can be collected for germination by placing an agar plate about 1 cm away from the fruiting surface. Spores will be shed for a week or more, provided the fruiting body is kept moist, and the fruit body will continue luminescing during that time. The bright greenish luminescence is strongest on the gill surface; in young fruit bodies the stipe is also luminous. In cultures on bread-crumb agar, the white fluffy mycelium becomes strongly luminescent after 2-3 weeks at 22°C, and remains bright for another 2 weeks. Stocks should be subcultured every 6 weeks. The spores are not luminous.

Because of its dense, even mycelial growth on agar surfaces, shadow photographs made solely by using the light emitted by *P. stipticus* make striking demonstrations. They can be made as follows: On a two-week old Petri dish culture, make a black paper cutout of any preferred motif, e.g., leaf, initials, etc. In a dark room, place a plate of panchromatic film, emulsion side down, over the Petri dish. After the minimum 3-hour exposure, a shadow image will have been made on the negative that can then be developed. The exposure required may be as long as 24 hours, depending on the light emission intensity and the sensitivity of the film. In fact, if no photomultiplier is available, this method can be used to determine large differences in light emission intensity, as in experiments to determine the effects of temperature, oxygen concentration, moisture or effects of various drugs.

Clitocybe illudens

Both the mycelium and fruiting body of *Clitocybe illudens* are luminescent in nature and the smooth white mycelium is luminescent on bread-crumb agar. It is known as the "Jack-o'-Lantern" mushroom, not only for its bright light but for its rich saffron-yellow pileus, which can be as much as 5" across. It can be found throughout the eastern United States from midsummer to autumn in large clusters on dying trunks and stumps of deciduous trees and has been reported to be poisonous. Mycelial cultures of this species also make good shadow photographs.

If an ultraviolet light source is available, exposure about 1 foot away for 1 hour will double the light output for a period of about 30 min in all species. The Petri dish cover or the flask cover must be removed during UV exposure.

See also Macrae, 1942; Harvey, 1952; Berliner, 1961b; McElroy and Seliger, 1962.

NUTRITION AND RELATED PHENOMENA

In the following section, arranged roughly by the taxonomic position of the experimental fungi concerned, are included a number of items that fall in the general area of nutrition, although not strictly confined to that subject. These are not intended as a complete coverage of the subject, but merely suggest the kinds of simple experiments that are appropriate for introductory mycology courses.

Spore Germination in Phycomyces

Relatively few asexual spores of this species germinate on synthetic media without prior special treatment. The presence of a natural product, such as yeast extract or peptone, in the medium increases germination without other special treatment.

To obtain abundant spores for use in this experiment, grow the fungus in 250-ml flasks containing a suitable broth medium for sporulation (M-82 + 5 g glucose and 1 g asparagine). Spores may be harvested from cultures 8-10 days old by adding 50 ml sterile distilled water and swirling to dislodge the spores. Divide the inoculum into two lots, in test tubes, keeping one as control and heating the other in a water bath at 50°C for 3 min with occasional agitation.

The above medium, plus agar, may be used for spore germination in Petri dishes. In preparing the medium, divide it into two lots and to one add 1 g/liter of potassium acetate. Pour 1 ml of heat-treated and unheated spore suspensions over both types of agar. Incubate near 25°C and observe for germination after 12, 24, and 48 hours. Compare percentages of germination under the four conditions tested. It is expected that the highest percentage will occur in heat-treated spores on medium containing acetate (Lilly *et al.*, 1962).

Thiamine Deficiency in Phycomyces

A liquid synthetic medium must be used in this experiment (M-82 less thiamine). In spite of using the purest-grade chemicals available, some trace of thiamine will be present and it is necessary to treat the medium with activated charcoal to remove it. Before adding the micro-elements or thiamine, add 5 g activated charcoal

(Norit) per liter, heat to boiling and filter through filter paper until clear. Add micro-elements, adjust the pH to near 6.0. Divide the medium into six lots and add thiamine to make the following concentrations in µg/liter: 100, 10, 5, 2, 1, 0. Dispense into clean Erlenmeyer flasks (25 ml in 250-ml flasks is convenient), cap with 50-ml beakers, or plug with cotton, and autoclave.

Inoculate with very small bits of young mycelium or with heat-treated spores (see preceding experiment) and incubate near 25°C. Cultures should show differences in growth rate after 2-3 days and maximum differences should be evident after about 5-8 days. Results should show that thiamine is an absolute requirement for mycelial growth and that more thiamine is needed for sporulation than for growth. If desired, the mycelium may be filtered, dried and weighed (Lilly and Barnett, 1951).

Choanephora cucurbitarum may also be used in this experiment.

Production of Carotene by Phycomyces

While this species produces carotene on most media favorable for growth, the amount of carotene produced is greatly influenced by nutrition and environment. A relatively simple favorable medium is liquid glucose (10 g) - ammonium sulfate (1 g). Thiamine is the only vitamin required. Adjust the pH to near 6.0, dispense 25 ml into 250-ml flasks and autoclave. Inoculate with bits of young mycelium or heat-treated spores and incubate near 25°C in continuous light or in alternating light and darkness. The bright yellow color, indicating the presence of carotene (mostly beta-carotene), should become evident about the fourth day and reach a maximum about the eighth day.

To demonstrate the effects of the medium on carotene production, add 2 g of potassium acetate to a second lot of medium and omit it from another. Also compare the amount of carotene with that produced on medium M-64.

The experimenter should examine some of the yellow hyphae microscopically and note that the carotene is located in the oil droplets; i.e., it is fat soluble.

The mycelium from a few cultures may be collected, the excess water removed by blotting and the pigment may be extracted with ether (Lilly *et al.*, 1960).

Production of Perithecia by Ceratostomella fimbriata

This species is deficient for thiamine, but it makes fair mycelial growth in low concentrations of thiamine. The amount of thiamine required for production of perithecia increases with an increase in concentration of glucose in the medium. Either liquid or agar basal medium may be used, as desired. The basal medium may be divided into four lots and the glucose and thiamine added in the following amounts per liter: 20 g - 100 µg; 20 g - 2 µg; 1 g - 100 µg; 1 g - 2 µg.

Inoculate with a few spores or bits of mycelium and incubate near 25°C for 7-10 days. Estimate the number of perithecia on each medium and the relative amount of mycelium. The total weight of cultures may be determined by filtering, drying and weighing. It is expected that a few or no perithecia will be formed in the medium high in sugar and low in thiamine (Barnett and Lilly, 1947b).

Biotin Requirements of Sordaria fimicola

An isolate known to be deficient for biotin should be used. Medium (M-82 + glucose 5 g and asparagine 1 g) should be treated with activated charcoal to remove traces of vitamins before adding micro-elements, thiamine, and desired amounts of biotin (5, 2, 1, 0.5, and 0 µg/liter). Agar will contain traces of biotin but usually not enough to interfere with the results.

Inoculate plates with bits of young mycelium and incubate near 25°C for 7-10 days. Observe the rate of mycelial growth, thickness of mycelium, color of mycelium, appearance and abundance of perithecia. Mount several perithecia from each medium in water and crush to release asci. Observe for maturity of ascospores and abnormal asci. Perithecia produced on media containing 0.5 µg biotin should show abnormal asci and ascospores (Barnett and Lilly, 1947a).

Note: The biotin requirements for growth of mycelium may be determined with greater accuracy if liquid media are used and dry weights are obtained.

Utilization of Carbon by Sordaria

This species will demonstrate that some fungi are capable of utilizing only certain carbon sources. *Sordaria fimicola* utilizes glucose, fructose, and maltose, but utilize little or no sucrose, lactose, and sorbose under most cultural conditions. Another test fungus should be compared in this experiment, such as *Glomerella cingulata*, or a species of *Penicillium*, *Aspergillus*, or *Fusarium*, which are capable of utilizing most of the sugars.

To the medium (M-82 + glucose 20 g and asparagine 2 g) add both thiamine and biotin. Agar medium can be used, but liquid media will give more accurate comparisons. For other carbon sources, substitute each test sugar for the glucose fraction.

Inoculate media with bits of young mycelium and incubate near 25°C for 6-10 days. Observe the cultures often to determine early growth on certain sugars. Obtain dry weights of mycelium grown in liquid media or measure diameters and thickness of colonies on agar. A good comparison of the two methods of measuring growth can be made if both liquid and agar media are used (Lilly and Barnett, 1953).

Mutualistic Symbiosis in Relation to Vitamins

Isolate of *S. fimicola* must be deficient only for biotin (not for thiamine). All known isolates of *P. blakesleeanus* are deficient for thiamine.

Add no vitamins to the medium (M-82 + glucose 10 g and asparagine 1 g). Liquid medium may be used, but the behavior of the two fungi is observed more easily on agar medium. A good grade of agar contains only traces of vitamins, not enough to affect the results of this experiment.

Inoculate with bits of young mycelium, placing the two species about 1-2" apart on agar plates. Use single-species cultures as checks. Observe frequently for early slow growth of mycelium of both fungi, followed by increased growth as the two species grow near each other and finally the sporulation of each in the area first occupied by other species. Each fungus synthesizes and excretes into the medium the vitamin for which the other species is deficient.

High Manganese Requirements

Selected species of test fungi are suggested here because of their high manganese requirements for normal growth or sporulation. Because of this it is not necessary to remove all traces of manganese from the basal medium in order to demonstrate its effects on these species. The basal medium (M-82 + glucose 5 g and asparagine 1 g) contains enough manganese for normal growth and sporulation of many fungi, e.g., *Glomerella cingulata, Trichoderma lignorum, Alternaria tenuis*. These species or others may be used as controls. Use *Chaetomium* spp. (*C. globosum, C. bostrychodes, C. elatum, C. convolutum, C. murorum*, or others) and *Neocosmospora vasinfecta* as test organisms on liquid or agar medium. Divide the basal medium into two lots and to one add 0.5 mg manganese per liter.

Inoculate with very small bits of mycelium so there will be little carry-over of manganese from the inoculum culture; incubate near 25°C. Observe frequently for early growth on some media and for differences in appearance. Measure colonies, or obtain dry weights of broth cultures, after 4, 8, and 12 days. Compare microscopic appearance of severely restricted mycelium on the two media. Observe difference in perithecia of *N. vasinfecta* on the two media.

Sufficient manganese is required for normal extension of hyphae of *Chaetomium* spp. and for formation of perithecia by *N. vasinfecta* (Barnett, 1964a).

Sporulation Following Different Methods of Inoculation

Species of *Melanconium, Pestalotia,* or *Gloeosporium* may be used to demonstrate rapid sporulation.

Prepare inoculum by collecting conidia and suspending in sterile distilled water. Inoculate the plates (M-64 + glucose 20 g and yeast extract 2 g) by two methods: (1) A small drop of spore suspension at the center; (2) by flooding the entire plate with 1 ml of a heavy spore suspension. A dilution of 1:10 or 1:100 may be used for comparison.

Observe the cultures daily for the production of spores. Use 10X and 43X objective of microscope to determine how spores are produced in the flooded plates. In these there is usually a complete lack of acervulus-like or sporodochium-like structures and spores are produced within 24-48 hours, directly on young hyphae or short conidiophores. Sporulation in point-inoculated plates is usually delayed for several days and localized in sporodochium-like structures (Timnick *et al.*, 1952).

Sugar Concentration and Conidial Size

Suggested test fungi are *Helminthosporium sativum* or *H. victoriae*. Some other species are not as satisfactory. Concentration of glucose in the medium (M-64 or M-82) should range from 0.5 to 40 g per liter, with water agar as control.

Inoculate plates with bits of young mycelium and incubate 10-14 days, allowing sufficient time for abundant sporulation. Make slides of spores from each concentration and measure length and count the number of cells in each of 25 spores from each culture.

Plot the results on a graph, using sugar concentration against average length of conidia. These results should emphasize the need for a standard medium for taxonomic studies of fungi in pure culture.

Note the method of formation of conidia at the apex of conidiophore, followed by new apical growing point of conidiophore, resulting in lateral appearance of older conidia (Elliott, 1949).

23 Fungus Genetics

GENETICS OF TRUE SLIME MOLDS

So far as is known, fusion of gametes (swarm cells or myxamoebae) is a prerequisite to plasmodial formation in all Myxomycetes. In some species, the gametes are of two mating types and plasmodia are not formed unless both mating types are present in the same culture. This may be demonstrated as follows: Secure relatively fresh sporangia of *Didymium squamulosum* and *Didymium iridis*. Make spore suspensions in 2 ml water and pour them on the surface of the medium (M-8b). Allow the agar surface to dry. Under the highest magnification available in a dissecting microscope, examine the surface of the agar and locate isolated spores. Using a fine insect pin mounted on a match stick, cut 20 small blocks of agar bearing *single spores* of each species and transfer them to cornmeal agar plates, one to a plate. Pour 2 ml of a suspension of *Enterobacter aerogenes* cells over the spore and incubate for 2 weeks. Examine the plates for presence of plasmodia. All cultures of *D. squamulosum* in which the spores have germinated (not all spores germinate) should produce plasmodia. Very few, if any, of the *D. iridis* cultures should have plasmodia.

Four lyophilized, single-spore-derived clones will serve as source material for further study of *D. iridis*. Among them they possess all of the necessary genetic characteristics for the experiments under consideration.

For convenience, the clones are simply numbered one through four, and the genetic markers that each possesses are as follows: #1 = A_1b; #2 = A_4b; #3 = A_4b; #4 = A_2B. The letter "*A*" refers to the mating locus, whereas "*B*" designates a locus that determines whether plasmodia will display the wild type brown color or the mutant cream color. By crossing these four clones in all possible paired combinations, six different crosses will be obtained. Because the mating system is a one-locus, multiple-allelic one, plasmodia should develop only in the five crosses heteroallelic for the *A* locus. Table 8 summarizes these results and also indicates the expected color of each plasmodium. Insofar as plasmodial color is determined by alternative dominant (*B*) or recessive (*b*) alleles at a single locus, it is of course expected that only the crosses that bring together two recessive alleles (*bb*) will be cream colored, while those carrying one dominant and one recessive (*Bb*) should be brown.

The ability of myxomycete plasmodia to migrate over surfaces is well known and can be taken advantage of in the laboratory. For example, by allowing two different plasmodia to migrate toward each other until they come into intimate contact, it can be determined whether they are compatible or not. If they are compatible, in a matter of minutes the two plasmodia fuse and become a single entity with a common protoplasm. On the other

TABLE 8 Inheritance of Plasmodium Color in *Didymium iridis*.

	1 (A_1b)	2 (A_4b)	3 (A_4b)	4 (A_2B)
1 (A_1b)	--	Cream	Cream	Brown
2 (A_4b)	Cream	--	--	Brown
3 (A_4b)	Cream	--	--	Brown
4 (A_2B)	Brown	Brown	Brown	--

hand, incompatible plasmodia do not fuse even when they remain in contact for extended periods of time. Using the five plasmodia referred to above in all possible paired combinations (Figure 21), ten different tests for compatibility can be made (Table 9). It happens that only two of these combinations can be expected to show fusion: 1x4(A_1A_2Bb) X 2x4(A_2A_4Bb) and also 2x4(A_2A_4Bb) X 3x4(A_2A_4Bb). The first combination obviously will result in production of a heterokaryon, because the nuclei of the two plasmodia differ at the mating type locus (i.e., A_1A_2 vs. A_2A_4). It is worth noting that if the heterokaryotic plasmodium is allowed to sporulate, the spores are presumably of three different kinds, A_1, A_2, and A_4. A closer look at Table 9 will show that six of the fusion tests will bring together a brown and a cream plasmodium. No fusions involving these two types are expected to occur, but in fact these pairings routinely create such color heterokaryons. For certain kinds of investigations, heterokaryons provide exceptionally useful material.

Although significant information on what determines plasmodial compatibility in *D. iridis* is available, the genetic basis of the system is not yet fully understood.

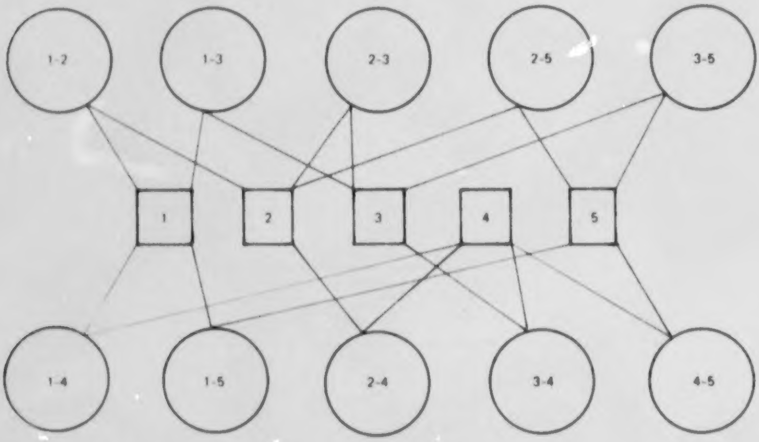


FIGURE 21 *Diagram of plasmodial fusions in all possible combinations.*

TABLE 9 Plasmodial Fusion in *Didymium iridis*.

	(A_1A_4bb) 1x2	(A_1A_4bb) 1x3	(A_1A_2Bb) 1x4	(A_2A_4Bb) 2x4	(A_2A_4Bb) 3x4
(A_1A_4bb) 1x2	-	-	-	-	-
(A_1A_4bb) 1x3	-	-	-	-	-
(A_1A_2Bb) 1x4	-	-	-	+	-
(A_2A_4Bb) 2x4	-	-	+	-	+
(A_2A_4Bb) 3x4	-	-	-	+	-

In order to cross the clones in the six combinations shown in Table 8, take several loopfuls of cell suspension from *two different clones* that are about 10 days old and put them together in a dish; add 3 ml of water. Macroscopic plasmodia should be evident in all fertile crosses in 4-7 days. One set of plasmodia should be left in a well-lighted room (but not in direct sunlight) without being fed. Sporangia probably will develop in these cultures when they reach an age of 10-12 days. The second set of plasmodia should be fed sterile pulverized Quick Quaker oats to insure their becoming large and vigorous. The oats should be sprinkled evenly over the surface of each plate but in sufficiently small quantities so that the agar surface is only about half covered. In 3-6 days following such a feeding, a plasmodium usually grows large enough to cover much of the agar surface area.

At this point it should be possible to distinguish between brown and cream-colored plasmodia. However, if there is any doubt, by means of a spatula cut out blocks of agar containing a vigorous portion of plasmodium from each dish and transfer, plasmodial side up, onto plates of freshly poured half-strength cornmeal agar (M-8a). After they have migrated off the blocks (usually within a few hours), examine them for color again and record your results.

To maintain plasmodia, transfer a vigorous portion of each every six days to fresh plates. Repeat the feeding procedures. One feeding after each transfer is sufficient.

Tests for plasmodial compatibility may be carried out by placing agar blocks containing vigorous plasmodial fans together on plates. These should be positioned about 2 cm apart and in such a way that the plasmodia will migrate toward each other. Ordinarily, it takes about 5 hours for the two plasmodia to collide, but length of time is extremely variable. As soon as the two plasmodia touch, they should be examined at the point of contact by transmitted light with a dissecting microscope. If they are compatible, in a matter of a few minutes common veins will be established between them, and the protoplasm will be readily seen to flow from one to the other. On the other hand, incompatible plasmodia may become superimposed, and the inexperienced observer could mistake this for fusion. However, whatever doubt there may be is dispelled when the two plasmodia pull apart as they eventually do if they are incompatible.

For establishing new single-spore clones, carefully lift a sporangium by its stalk with a pair of fine-pointed forceps and place it in a deep slit made in the agar in a Petri dish. Add 3 ml sterile distilled water. Lift the cover just enough to allow entrance of your forceps, and then squeeze the sporangial head. Replace the cover and gently rotate the dish to distribute the spores. Now streak several loopfuls of this suspension onto the surface of a second dish. By carefully adjusted transmitted light through a dissecting microscope, at a magnification of 60X or higher, the individual spores should be recognizable. Using an appropriately fine-pointed instrument, cut out a small block of agar containing only one spore and transfer it to M-8a. Add a drop of a very dilute water suspension of *Escherichia coli* to this plate; then flood the surface with 3 ml sterile distilled water. Incubate at 23°C or in a cool room. Viable spores should yield populations of amoebae in about a week.

Alternatively, one can with fine-pointed forceps crush a single sporangium on the side of a test tube containing 10 ml of sterile distilled water, thus making a dilute spore suspension. Pipette 10-15 drops of this suspension onto the surface of a half-strength cornmeal agar plate (M-8a). Tilt the plate so the suspension will be evenly distributed over the surface and allow to dry. Individual spores are then isolated as above.

See Collins, 1963, 1966; Alexopoulos and Koevenig, 1964; Collins and Ling, 1964; Collins and Clark, 1966a,b.

HETEROKARYON FORMATION IN PHYCOMYCES

In *Phycomyces*, the hyphae stop growing shortly before they meet. Thus, the lack of hyphal fusion leads to the absence of heterokaryon formation in nature, unless through sexual fusion between gametangia of opposite sex or spontaneous mutation in hyphal cells. However, two techniques have been known to produce artificial heterokaryons: (1) Based on a very early experiment in which the tip of a decapitated sporangiophore was inserted into a genetically different sporangiophore and the cell contents injected; and (2) based on Weide's (1939) technique, whereby heterokaryotic sporangiophores were obtained on a mixture of cell contents that were squeezed out of two different mutant sporangiophores. The experiment outlined here was designed to obtain heterokaryotic sporangiophores more readily by employing a grafting technique.

Phycomyces blakesleeanus mutants, strain C9 (-), accumulating lycopene, and strain S5, which is albino, were separately cultured under sterile condition on 1.5% Sabouraud's glucose agar in 5.4-cm Petri dishes kept in a chamber consisting of a 10-cm Petri dish covered by an inverted 400-ml beaker. Stage 1 sporangiophores, growing straight, were plucked from mycelia of different strains and placed head to head on small blocks of 1.5% Difco purified agar that were arranged on glass slide. After both sporangiophores were decapitated with alcohol-sterilized iris scissors (Vannas) dipped in sterile distilled water before use, the blocks were carefully pushed together under a dissecting microscope until the open cut-ends came in contact (Figure 22). No insertion of one end into the other was performed. The glass slide carrying the grafts was placed on U-shaped glass rod in a Petri dish bottom with a shallow layer of distilled water, and an inverted crystallizing dish, 5 cm in height, was placed into each dish as a cover. The grafted specimens were cultured under room conditions. After about 1 hour, the drop of water that frequently appeared on the graft union was carefully removed with tips of forceps without touching the plants. The success of a graft was judged by the formation of a new cell wall between two sporangiophore portions and by fusion of cell contents at the graft region.

At 12 hours after grafting it was found that about 39% of all grafts were successful (Table 10). It is known that, in almost all decapitated sporangiophores, regeneration occurs at or very close to the cut end; this was also the case in the grafts, in that the initials of regenerated sporangiophores usually appeared around the graft region. Since strain C9 formed red sporangiophores and sporangia because of lycopene accumulation, and strain S5 produced white sporangiophores bearing white sporangia because of lack of carotenoid synthesis, whenever the regenerated sporangiophores were heterokaryon they were easily recognizable by their color under a dissecting microscope. One can expect the sporangiophores to be heterokaryotic when a single sporangiophore, which may be of joint origin, appears at the graft region (Figure 22) or when the sporangiophores containing the cell contents of a red mutant regenerate on a white mutant sporangiophore. The regenerated sporangiophores and sporangia are sometimes clearly yellow, indicating the complementation in the β-carotene production.

Spores that mature on possibly heterokaryotic sporangiophores developing from successful grafts can be plated on acid agar media containing glucose, asparagine, and yeast extract to get colonial growth for the genetic analysis. The colonies then appear of different color shades, from white to red, according to the proportion of two types of nuclei, and in close approximation to theoretical values. Thus, sporangiophores are obviously

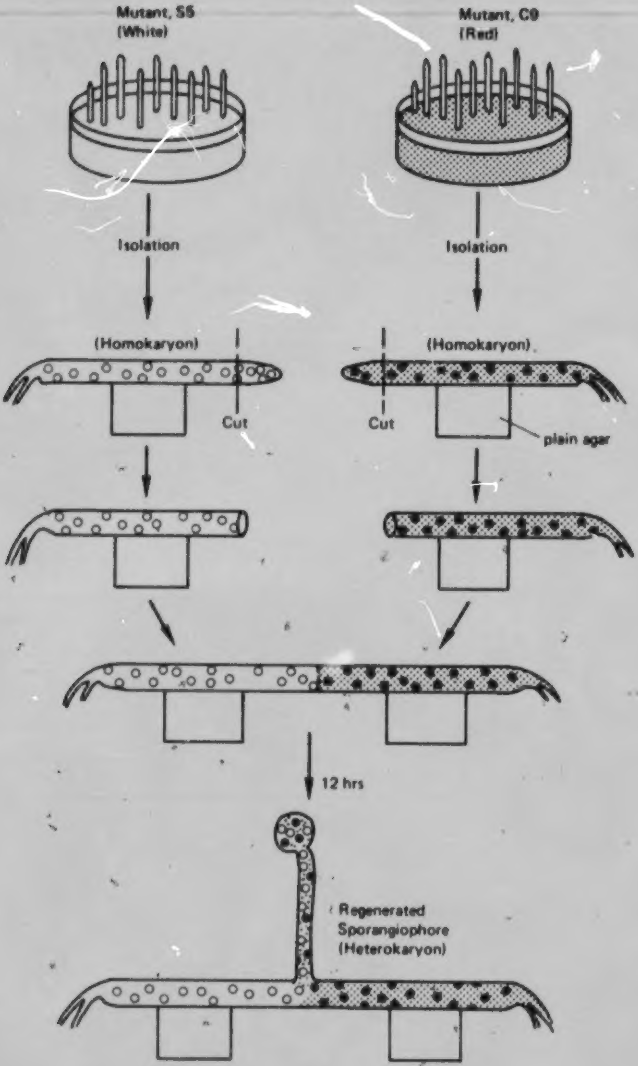

Mutant, S5
(White)
Mutant, C9
(Red)
Isolation
Isolation
(Homokaryon)
(Homokaryon)
Cut
Cut
plain agar
12 hrs
Regenerated
Sporangiophore
(Heterokaryon)

FIGURE 22 *Grafting of Phycomyces mutants.*

TABLE 10 Frequency of Successful Grafts Between C9 and S5 Mutant Sporangiophores.

Experiment	Total Number of Grafts	Number of Successful Grafts	Number of Unsuccessful Grafts
I	50	16	34
II	63	22*	41
III	55	34	21
IV	49	12	37
Total	217	84 (38.7%)	133 (61.3%)

*Seventeen sporangiophores heterokaryotic.

heterokaryotic, though a great variation is found in the nuclear proportions.

GENETICS OF *SORDARIA* AND *NEUROSPORA*

Sordaria and *Neurospora* have proven useful for demonstrating Mendelian segregation, partly because these fungi are readily grown in the laboratory and complete their life cycles in a week or so and partly because all the products of meiosis are arranged in an orderly fashion that reflects the precise order of meiotic events. Species of *Neurospora* have been studied genetically in more detail than any other fungi, but they produce large numbers of macroconidia that are a troublesome source of contamination in the laboratory. For this reason and because a number of readily analyzable visible mutants are available in *Sordaria*, species of this latter genus are recommended here.

Sordaria fimicola is a homothallic species that lacks microconidia, while *S. brevicollis* is heterothallic and produces microconidia. The normal ascospore color in both species is dark brown, the pigments being located in the spore wall. A number of ascospore color mutants have been obtained in both species that permit genetic analysis of crosses by direct examination of the hybrid asci, thus circumventing the necessity of ascus dissection. Most of the experiments included here involve direct visual analysis of the asci. The text, *Fungal Genetics*, by Fincham and Day (1971) is highly recommended as a general reference.

Both *S. fimicola* and *S. brevicollis* are maintained and studied on an agar medium that will hereafter be referred to as CM + agar (M-9). The minimal medium for *Neurospora* is also satisfactory for wild type cultures of *Sordaria*. Crosses are made in standard-size Petri dishes, the inocula generally being placed about 4 cm apart on the agar surface. An alternative method may be preferred for *S. brevicollis*, as it tends to yield a larger number of perithecia. This involves microconidiation of one mating type by the other.

While crosses may be grown at room temperature in the laboratory, it is recommended that they be maintained in an incubator at 23°-25°C. Light is not necessary. Mature perithecia of *S. fimicola* develop in about 7 days, those of *S. brevicollis* in about 10 days. After the perithecia have matured, development may be greatly slowed down and the cultures kept for class use during an additional week by placing the plates in a refrigerator.

When ascospore germination is desired, very high percentages of germination may be obtained, usually within 6-8 hours at 30°C, by placing the spores on CM + or plain agar containing 0.7% sodium acetate (and 3-4% agar when extra firmness is desired). If one wishes to do ascus dissection, this agar is also very satisfactory in keeping down bacterial contamination, since it is highly acid. Also, the acetate tends to weaken the ascus wall, which is an advantage if the dissection is done soon after the asci are transferred to the agar, but after an hour the ascus walls become too hydrolyzed for accurate dissection. In transferring asci for dissection, the hybrid cluster pressed out of the perithecium may be picked up on the tip of a sharp needle and placed in a drop of water on the agar surface. As the drop dries out, the asci will spread out into a nice rosette ready for dissection. While a micromanipulator is a great help in dissecting asci, a number of investigators have become very adept at dissecting by hand, although it requires patience and practice.

As previously noted, direct observation of hybrid asci is simple. First, several perithecia are scraped from the surface of a culture with a flat-tipped or arrowhead needle and placed in a drop of water on a slide. A cover slip is placed on top and pressed down sufficiently to expel the ascus clusters from most of the perithecia without squashing the asci. The cover slip is then removed, any adhering material being scraped back onto the slide. Under a dissecting microscope, several clusters of hybrid asci are pulled to one side into a nearby fresh drop of water, thus freeing them from perithecial walls and other debris in the original drop. A cover slip is then gently placed over these clusters, which causes them to spread out into rosettes in which the asci may readily be observed and classified. If the asci appear to become overly squashed during prolonged examination, they may be restored to normal by the addition of a little water at the edge of the cover slip. For best results in class, the data obtained by all the students should be pooled for final computation so that each student may compare his own figures with the usually more accurate ones obtained from the larger amount of pooled data.

Cultures of *Sordaria* may be maintained from year to year in tubes of CM + agar (preferably in sporulating condition) at a temperature of around 7-10°C. They need not be transferred more often than twice a year. Screw-cap tubes are preferable, as they prevent the agar from becoming desiccated.

Mendelian Segregation

One of the first points that becomes clear in an examination of hybrid asci in a simple cross such as that involving the gray-spored mutant (*g*) and wild type (g^+) cultures of *Sordaria fimicola* is that the hybrid asci segregate in a 4:4 ratio for spore color. In other words, at meiosis the mutant locus on its chromosome segregates from its counterpart, the wild-type allele, on its chromosome, and each locus is replicated the same number of times during the three divisions (the third being equational) in the ascus so that the mature ascus contains four gray spores and four wild-type spores.

In analyzing the results of these crosses it is helpful to keep in mind that *Sordaria fimicola* is a homothallic species that produces ascogonia but apparently not antheridia (Figure 23). In a cross, the two types of nuclei (e.g., g and g^+) become associated by nuclear migration through hyphal anastomoses. Hybrid perithecia appear to have their origin in heterokaryotic ascogonia that arise from heterokaryotic vegetative hyphae. However, in this species any combination of nuclear pairing is equally possible, and all combinations are fertile. Therefore, along the line of contact between two cultures such as gray-spored and wild type, one finds not only hybrid perithecia but selfed perithecia of both parental types. Also, in some of the hybrid perithecia there appear homozygous asci of either parental type in addition to the hybrid asci. Since nuclear migration in these matings does not generally extend far from the line of contact, hybrid perithecia are likely to be found only near this line.

Gene-to-Centromere Distances

The crosses used to illustrate gene-to-centromere determinations involve the gray-spored and tan-spored mutants and the wild type of *S. fimicola*.

Wild Type x Gray-Spored An examination of clusters of hybrid asci from this cross quickly reveals that there are a good many more asci that show 2:4:2 and 2:2:2:2 arrangements of spore pairs than there are those that have a 4:4 arrangement. The pairwise arrangement results from segregation at the second meiotic division. That is, because of a crossover between the centromere and g (gray-spored) locus involving a pair of homologous chromatids at meiotic prophase, the g^+ and g alleles do not segregate until the second meiotic division (MII)

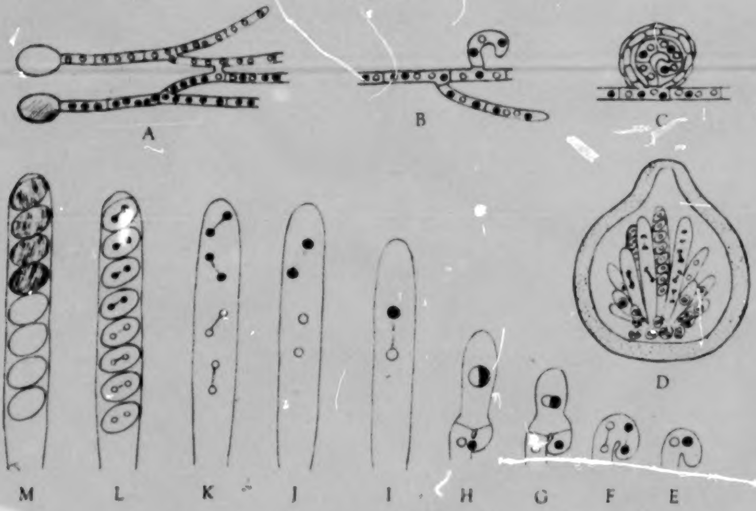


FIGURE 23 *Diagram of perithecial formation in Sordaria fimicola, showing origin of asci heterozygous for a spore color marker.* A. *Origin of heterokaryon.* B. *Origin of heterokaryotic ascogonium.* C. *Perithecial initiation.* D. *Maturing perithecium.* E. *Crozier.* F. *Conjugate nuclear division in crozier.* G *and* H. *Karyogamy in young ascus.* I. *First meiotic division.* J. *Second meiotic division.* K. *Third (equational) nuclear division.* L. *Mitosis in spores.* M. *Mature ascus with four wild type and four light ascospores.*

(Figure 24). Lack of crossing over between the locus and centromere results in the 4:4 arrangement indicative of segregation in the first meiotic division (MI). Both the 2:2:2:2 and 2:4:2 arrangements are equally probable because of randomness in orientation of g and g^+ chromatids on the second division spindles. Occasional irregular spore arrangements such as 3:1:1:3 (a disarranged 4:4 type) will be encountered but are not common enough to affect the data seriously, and, in fact, most of them are readily interpreted as MI or MII segregations. They are probably due to occasional spindle overlap in the third (equational) nuclear division in the ascus.

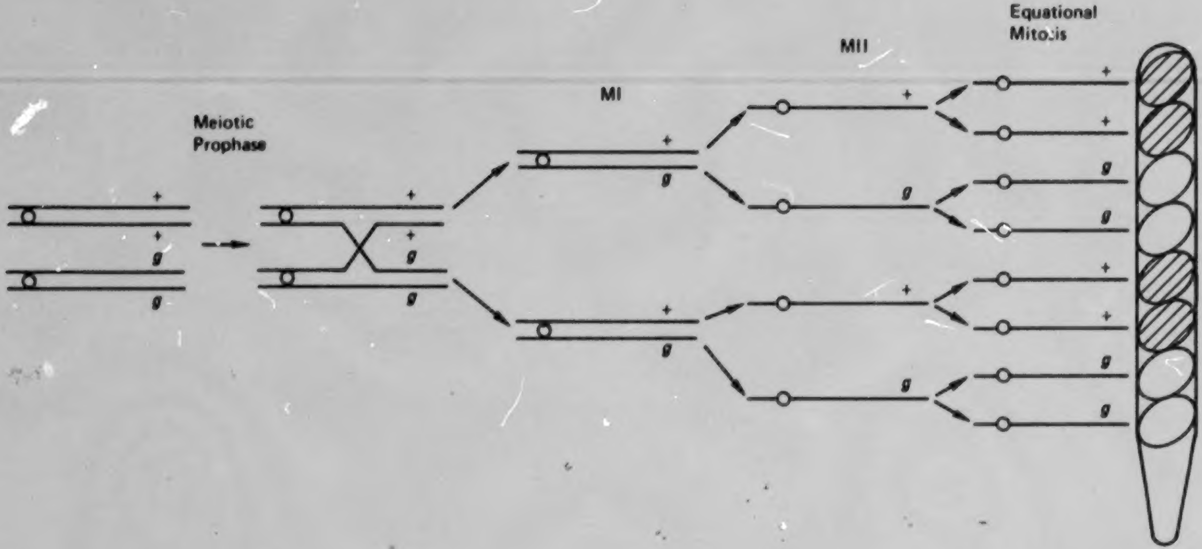

Meiotic Prophase
MI
MII
Equational Mitosis
+
+
g
g
+
+
g
g
+
g
+
g
+
g
+
g
+
+
g
g
+
+
g
g

FIGURE 24 *Behavior of a tetrad of chromosomes heterozygous for the gray-spored factor. Note that a 2-strand crossover has occurred in prophase, resulting in segregation at the g locus in the second meiotic division* (MII).

When the asci from this cross are counted, it will be found that the proportion of MI:MII asci is around 1:2, the MII asci, therefore, comprising about two-thirds of the total. This means that a great deal of crossing over has been taking place between the locus and centromere and that the locus is a considerable distance from the centromere. The closer a locus is to the centromere the shorter will be the interval between them in which crossing over can occur and, accordingly, the lower will be the proportion of MII asci. If the locus were at the centromere, there would be no crossing over, and all the asci would be MI. Conversely, the farther the locus is from the centromere the longer will be the interval in which crossing over can occur, and the greater the proportion of MII asci, up to a point (67%).

Relative distances between loci and their centromeres are measured in *map units* (or Morgan units), which in turn are determined according to the frequency of crossovers in the gene-to-centromere interval. Originally, map distances were determined in such organisms as the fruit fly, and of necessity had to be based on random analysis of meiotic products among the progeny rather than on percentages of tetrads as in the present experiments. In *Sordaria*, each MII ascus represnets a tetrad of chromatids in which there occurs a single exchange involving only two of the four chromatids. Therefore, in order to obtain a map distance comparable to that applying to higher organisms, we must divide the percentage of MII asci by two. Thus, if 20% of the asci were of the MII type, the gene-to-centromere distance would be 10 map units.

Since the proportion of MII asci in the cross g^+ x g is 66.7%, it might be expected that the g locus is 33.3 map units from the centromere. However, published studies with intervening markers have shown that g is about 60 units from the centromere. The discrepancy is explained as follows: As the distance between locus and centromere increases, not only is there a proportional increase in single crossovers, but there is simultaneously an increased probability of additional crossovers in the same interval. Asci resulting from two-strand or four-strand double crossovers will appear as MI asci (as though no crossing over had occurred), while three-strand doubles will produce the MII arrangement. Thus, a point on the chromosome is eventually reached beyond which there will be no further increase in the MII class. This point is attained at about 33.3 map units, at or beyond which the percentage of the MII class will remain approximately 66.7%.

Wild Type x Tan-Spored This cross allows a direct computation of the distance of the tan-spored mutant locus from the centromere. Direct counts of hybrid asci will reveal that about 56% of them segregate at the second meiotic division. Dividing this percentage by two, we find the distance of the *t* locus from the centromere to be around 28 map units.

Linkage Relationships

If two loci can be demonstrated to occur on the same or homologous chromosomes, they are said to be linked, in which case they will not segregate independently at meiosis. If the two loci are on different chromosomes, they will segregate independently. In crosses heterozygous for two mutant loci, three types of hybrid asci are produced, and the proportions of these types indicate whether the two loci are linked or independent. For example, if we cross the gray and tan mutants used in the foregoing experiments ($gt^+ \times g^+t$), we obtain the following three types of asci: *parental ditype* (PD) with four gray and four tan spores, *recombinant ditype* (RD) with four wild type and four double-mutant (*gt*) spores, and *tetratype* (T) with two gray, two tan, two wild-type, and two double-mutant ascospores. The arrangement of the spore pairs in the ascus is unimportant to the present determination.

If loci *g* and *t* are on nonhomologous chromosomes and are therefore inherited independently of each other, the number of PD and RD asci should be equal. If the two are linked on the same chromosome, then it is clear that a special type of crossing over (four-strand double crossovers) would be required to produce an RD ascus, while a single crossover would produce the tetratype (T).

Clearly, then, RD asci will be fewer in number than PD, usually strikingly so unless distant linkage is involved. At the same time, the percentage of T asci will indicate the distance (within the limits previously described) between the loci, just as the percentage of MII asci in the foregoing experiments indicated the distance of a locus from the centromere.

Gray-Spored x Tan-Spored An ascus count here quickly reveals that PD and RD asci occur in equal proportions, indicating an absence of linkage. The T asci are very abundant but of no particular interest if linkage is not involved, and therefore no further analysis is needed. The two loci, as indicated by this test, are indeed on nonhomologous chromosomes.

Gray-Spored x Restricted-Growth Mutant The restricted growth mutant (r_1) has a distinctly slower vegetative growth rate than the wild-type culture, but the locus has no effect upon spore color. Therefore, in analyzing the asci to determine whether there is linkage with g, the spores need to be dissected out and the types of asci (PD, RD, T) recorded. Since both members of each spore pair are genetically alike, it is sufficient to dissect out one member of each pair for analysis (or to remove the spores in four successvie pairs). This should be done on firm CM+ agar with sodium acetate as described above. After separation an adequate distance apart on the isolation plate, with about 6-8 hours allowed for germination to begin, the four spores (or spore pairs) may then be cut out on small individual blocks of agar with a sharp arrowhead needle and transferred to a plate of CM+ agar, where they are numbered in order of occurrence in the ascus and the spore color recorded. Three or four lines of spores representing as many asci may be placed on a single Petri dish, if properly spaced. Within 2 days it will be possible to score the asci completely, as differences between restricted and wild-type cultures become readily apparent within this time. When the information on rate of growth is combined with the spore-color character, it will be found that there are very few RD asci in proportion to PD asci, demonstrating that the loci g and r_1 are linked. A tetratype percentage of around 26% shows that the two loci are about 13 map units apart.

If ascus dissection proves too difficult, random spore analysis can be used to demonstrate linkage between r_1 and g, if a few precautions are taken. One must first select one or more ascus clusters that contain *only* hybrid asci and no homozygous ones. With a small pipette these may be transferred to CM+ acetate agar, where they are left undisturbed for about an hour to allow the acetate to weaken the ascus walls. Now, if a small drop of water is added and the mass of asci gently macerated with the blunt end of a stirring rod, most of the spores will be freed. A few milliliters of water are then added to the surface of the dish and swirled about to disperse the spores over the agar surface. The dish is then left in a warm place (in an incubator at 30°C or under a lamp) to improve germination, during which time the surface water will also evaporate.

Germination will begin in about 6-8 hours from the time the spores were placed on the acetate medium. At this time single germinating spores are cut out on small blocks of agar and transferred to plates of CM + agar, or to CM + acetate agar if sterile technique has not been carefully followed. A dozen or so spores properly spaced may be placed on a single dish. At the time of transfer the color of each spore is recorded. Within 2 days it will be possible to distinguish the restricted mutants from the more rapidly developing wild-type mycelia. When spore-color and growth-rate data are combined, it will be found that spores of parental genotypes (gr_1^+ and g^+r_1) are much more common than those of recombinant types ($g^+r_1^+$ and g_1r_1), thus demonstrating linkage. The percentage of recombinant spores will be on a par with figures obtained by random analysis in higher organisms, and therefore the percentage of recombinants (about 13%) is the direct measure of the distance (13 map units) between the two loci.

Beige-Spored x Yellow-Spored This cross involves mutants of the heterothallic species, *Sordaria brevicollis.* The mating type factors, *A* and *a*, are enclosed in parentheses, since they do not enter into the following genetic analysis. While this species would at first appear ideal for genetic analysis, particularly in view of its production of hybrid asci only, there are certain inherent disadvantages. *S. brevicollis* is prone to give rise to occasional spontaneous mutants with an orange mycelium, and crosses homozygous for the orange factor (linked to mating types) tend to be sterile. Secondly, there is frequently an overlapping of spindles in the second meiotic division that distorts the relationship between MI and MII asci (but which may easily be corrected). For purposes of linkage determination, however, the latter is of no importance and may be ignored.

While perithecia may generally be readily obtained by pairing mycelia of opposite mating type on Petri dishes, the best method for maximum production of fertile perithecia in this species involves microconidiation. It is suggested that for this particular cross a plate of CM+ agar be inoculated in about 4 places (more or less equally spaced) with the yellow-spored culture, $y_{10}(a)$. At the same time, a beige-spored culture, $b_4(A)$, is started on CM+ agar containing 0.7% sodium acetate. The acetate greatly enhances microconidial production. When the cultures are 4 days old, place 2 ml of sterile water on the surface of the beige-spored culture and swirl around to loosen the microconidia, then pour the suspension over the surface of the yellow-spored culture and again swirl the water over the surface. Within 6 days after microconidiation, mature hybrid perithecia will be present. By this method the protoperithecia of the yellow-spored culture are fertilized by the microconidia of the beige-spored culture.

A direct examination of the asci from this cross reveals the expected three types, but it becomes immediately apparent that PD asci far outnumber the relatively few RD asci, thus demonstrating that the loci are linked. A count of tetratype asci will show that they comprise about 18% of the total, thus demonstrating that the loci are approximately 9 map units apart. The pair of very light spores in tetratype asci and the four in RD asci are double mutant ascospores.

Heterokaryon Complementation

A common method of demonstrating heterokaryon complementation in fungi is to inoculate two nonallelic nutritional mutants together onto plates of minimal medium. Numerous nutritional (or biochemical) mutants of such fungi as *Neurospora crassa* and *Aspergillus nidulans* are available in various laboratories, probably the most extensive being the stock of *Neurospora crassa* cultures maintained by the Fungal Genetics Stock Center at Humbolt State College, Arcata, Calif. 95521.

The effective demonstration of complementary heterokaryon development with such mutants depends upon the use of purified culture media that contain a minimum of contaminating growth substances. The preparation of such media in the laboratory can be laborious and time-consuming, and the cost of commercially purified agar is high. Fortunately, there is a simpler approach to this problem.

Heterokaryon complementation of the type described by Dodge as "heterokaryotic vigor," involving the pairing of nonallelic dwarf mutants of *Neurospora tetrasperma*, may be readily demonstrated in *Sordaria fimicola*. One simply places together (in contact with each other) on plates of CM+ agar inocula of two nonallelic restricted growth mutants similar to the one (r_1) described earlier. The exogenous supply of growth substances in the medium does not assist these mutants to overcome their deficiencies. However, as the two inocula begin to grow out, hyphal anastomoses occur between them, and hyphae containing mixtures of both types of nuclei are produced. These hyphae now grow out rapidly like wild-type cultures. The phenotypically wild-type outgrowths may require several days to appear. Later, fertile perithecia will appear abundantly on them.

The combination used as an illustration here is the following: $g^+r_5 + gr_6$. When the perithecia mature on the heterokaryotic mycelium that forms, they will be found to be of the usual three types with regard to spore color, that is, the two selfed parental types (either wild-type dark-spored or gray-spored) and hybrid. The distribution of these types over the surface of the heterokaryotic mycelium, of course, gives a means of demonstrating the widespread occurrence of both types of nuclei in the mycelium.

While supplements in the medium do not enable these mutants to overcome the growth restrictions imposed by their mutant genes, normal supplementation does occur through the interaction of the two kinds of nuclei in a common cytoplasm. This is because each type of mutant nucleus carries the wild-type allele for the mutant locus of the other, and, therefore, whatever growth-influencing substance is lacking in one type of nucleus is compensated for by its synthesis in the other. Thus a phenotypically wild type heterokaryon develops. The deficient substances have not been identified.

In view of these observations, our heterokaryotic combination may be more accurately formulated as follows: $g^+r_5r_6 + gr_5^+r_6$. This relationship demonstrates that, while a heterokaryon is not a true diploid condition, it does enjoy some of the benefits of the diploid. The ability to form heterokaryons is undoubtedly of survival value to both sexually and non-sexually reproducing fungi.

See Olive, 1956, 1963; Carr and Olive, 1958; El-Ani *et al.*, 1961; Olive and Fantini, 1961; Chen, 1965; Chen and Olive, 1965.

SEXUAL AGGLUTINATION IN YEASTS

Some species of protozoa, algae, and fungi have complementary substances on their cell surfaces that cause opposite sexes to adhere when they come in contact. Zygote formation is stimulated. The process is called sexual agglutination. Some heterothallic yeasts are sexually agglutinative; others are not. The reaction is weak in primitive haploid species and stronger in diploid species. In one diploid species the reaction is strong whether the opposite sexes have been grown separately on solid media or in liquid media, and then brought together. In other diploid species it is strong only if the yeasts have been cultivated on solid medium or in liquid medium, but not by both cultural procedures.

Saccharomyces kluyveri

Saccharomyces kluyveri gives strong reactions whether cultured in liquid or on solid medium, and it is the species recommended for most studies. It sporulates abundantly as found in nature. Ascospores may be isolated and germinated; their progeny are easily maintained as unisexual cultures. Each sex self-diploidizes to a small extent to produce unisexual diploids and probably, eventually, higher ploidy states. The higher the ploidy of the individual isolates used, the larger their cells and the more strongly they agglutinate. Mating types commonly used are presumed to be triploid or tetraploid, and their zygotes will sporulate poorly if at all; bisexual cultures of some strains are available that sporulate abundantly.

The opposite sexes and bisexual cultures may be kept on agar slants or by lyophilization. When needed for demonstration, they should be transferred daily about three times, after which they will be observed to produce a strong reaction when approximately equal volumes of the two sexes are removed from the slants and mixed on an agar surface. Small amounts of cells from two slant cultures and from the mixture may be transferred to water. Macroscopically, the cells of the individual sexes will be observed to remain in suspension; the mixed cells will be agglutinated in clusters often containing millions of cells.

For classroom demonstration, inoculate flasks containing liquid yeast extract-malt extract medium (M-69) with cells actively growing on M-69 slants. Shake at 25-28°C or at room temperature for 48-72 hours. Pour equal volumes of the cultures into a large graduated cylinder, leaving about one-third of it empty. Place hand over open end, and invert about 20 times. Stop after first, second, fifth, and tenth inversion to observe degree of agglutination. The agglutination should be practically complete; within a few minutes after this mixing of the cells, the supernatant should be almost as clear as uninoculated medium. Note the pleasant odor. If 3% of glucose or sucrose is used in the medium, the increased amount of sugar yields more cells and consequently more abundant flocculation when cultures are mixed.

Using agar-grown cells, make a mixture of opposite sexes on an agar slant or plate. Spread some of the thoroughly mixed cells thinly on M-69 agar. Conjugation should start in about 1 hour and increase rapidly thereafter. If haploids or unisexual diploids are used, sporulation may be abundant, but if mating types 3 and 26 are selected, there may be little, if any, sporulation. Incubate for the production of ascospores at 25°C.

The best way to keep yeast cultures is by lyophilization. They may also be kept on slants, transferring every few months. The fresh slants may be incubated 1 or 2 days at 25-28°C or at room temperature, and then refrigerated.

See Wickerham, 1956, 1958; Wickerham and Dworschack, 1960; Wickerham and Burton, 1962.

Hansenula wingei

The haploid mating type strains 5 and 21 of the yeast *Hansenula wingei* exhibit a strong sex-specific agglutination reaction when mixed. After agglutination the solution clears dramatically as the cell clumps settle out. Agglutination is measured by determining the reduction in turbidity of a cell suspension after agglutination and settling of the clumps.

The haploid mating types can be maintained indefinitely in culture and conjugation between haploid cells will occur when the two strains are mixed together in synthetic liquid conjugation medium (M-85). The diplophase can also be maintained indefinitely in culture, since sporulation of diploid cells will occur only on special sporulation medium (M-36). Haploid strains can be recovered from the sporulated diploid in two ways. Single ascospores can be isolated by micromanipulation or by a heat treatment of sporulated diploid culture, since heating kills the vegetative diploid cells but not the more heat-resistant ascospores. After this heat treatment, the haploid cells can be isolated by single colony isolation.

The two strains, 5 and 21, exhibit different chemical sensitivities. For example, trypsin digestion destroys the agglutinability of strain 21 but not strain 5. This differential sensitivity of the two cell types to trypsin digestion is probably a reflection of the mode of attachment of the two agglutination factors to the cell wall. Trypsin releases the agglutination factor from the cell surface of strain 21 and consequently destroys its agglutinability. On the other hand, the agglutination factor from strain 5 is not solubilized by trypsin treatment although it is inactivated when in solution.

Grow the yeast cells in liquid complex medium (M-62) in an Erlenmeyer flask filled to only one-fifth capacity overnight at 30°C on a rotary shaker at 200-300 RPM. Use a 1% liquid inoculum or a loopful of cells from a stock slant into 25 ml of complex medium in a 125-ml Erlenmeyer flask. If lyophilized cells are received, wipe the outside of the ampoule off with 95% ethanol, break the top off, flame the opening, shake the dried cells into 25 ml of complex medium and grow as described above. To prepare stock slants, take a loopful of cells from liquid culture or from another stock slant and spread the cells over the surface of a slant of complex medium. Optimum temperature for growth is 30°C, but room temperature may be used if a 30°C incubator is not available. Incubate slants for 2 days at 30°C and then refrigerate for storage.

Agglutination Assay Suspension of cells of strain 5 and strain 21 are prepared by washing the cells in water, centrifuging, and resuspending the cells in agglutination buffer (SR-25) to a density of about 5×10^8/ml, which is the number of cells/ml at stationary phase. The cell densities of both mating types should be the same. The agglutination reaction is done by mixing 0.1 ml of each cell suspension of strains 5 and 21 together either on a wrist-action shaker for five minutes or on a vortex mixer for ten seconds. Standardize conditions of mixing so that within any series of comparative assays, all tubes are mixed the same length of time. Estimate the degree of agglutination either visually or by adding 5 ml of agglutination buffer, inverting the tube to mix, allowing agglutinated clumps to settle for

20 min and reading the remaining turbidity (O.D.) at 640 mμ by inserting the assay tube directly into a "Spectronic 20" spectrophotometer or an equivalent colorimeter. As controls, 0.2 ml of each cell suspension is treated separately as above and the O.D. at 640 mμ is determined in matched colorimeter tubes. The O.D. readings of the two controls are averaged to determine what the control level of turbidity would be if the cells had not agglutinated. The controls should give an O.D. reading of approximately 0.6. Strong agglutination should result in a reduction in the O.D. reading to below 0.1. Agglutination can be expressed as the percent reduction of turbidity from the control.

Conjugation Assay To quantify the formation of conjugal pairs, prepare cells of strain 5 or strain 21 for assay in the following manner: Grow each cell type in liquid culture just into the stationary phase (cells from very young cultures do not conjugate as well); wash the cells twice in water and resuspend the cells in conjugation medium to a cell density of about 5×10^8/ml. Sterile technique is not necessary during the conjugation assay. Determine the cell density in each suspension by using a Petroff-Hausser counting chamber or hemocytometer. Count a budding pair as one. To avoid cell counting, the suspensions can be approximately adjusted to the same cell number by turbidity measurements. Mix equal numbers of each mating type together. Immediately withdraw a 0.1-ml sample of the mixture and add the sample to 1 ml of 8 M urea (zero-time). Put the flask containing the mixed suspension on a fast rotary shaker. Observe the immediate agglutination reaction and then later observe that this reaction has increased in intensity. Withdraw 0.1-ml samples at half-hour intervals and add to 1 ml of 8 M urea. At the end of the experiment (after 5 hours), autoclave the samples in urea for 5 min. The 8 M urea stops the reaction and also serves to keep the unmated cells deagglutinated after autoclaving.

Put a drop of the autoclaved cell suspension in urea on a glass slide and cover the drop with a cover slip. Choose several representative microscopic fields for the conjugation counts and count all the cells in each field. Count more than 100 cells or conjugants per assay.

Conjugants usually form a distinctive dumbbell shape. If a phase microscope is available, conjugants can be counted directly. If a light microscope is to be used, the samples should be stained with an appropriate dye (e.g., methylene blue). To facilitate the calculations, make a differential count of the number of conjugant pairs alone, and the number of individual cells plus the number of conjugant pairs together. From these totals, the number of unconjugated cells is obtained by difference, and the number of single cells that conjugated is calculated by multiplying the number of conjugants by two. To calculate the conjugation percentage, the number of single cells that had conjugated is multiplied by 100, and this figure is divided by the number of single cells that had conjugated plus the number of unconjugated cells.

If the conjugation percentage is plotted vs. time, it should be found that there is a 1-hour lag, after which time the number of conjugants rises steadily until at 5 hours a maximum is reached at which about 80% of the population has mated. There is a minor fraction of the population that is physiologically unable to mate under these conditions. The 1-hour lag is sometimes abolished by the addition of malt extract or yeast extract to the conjugation medium.

Isolation of a Diploid Hybrid The two mating types can be mated by mixing a loopful of each together on an agar plate and incubating the plate overnight at 30°C, using sterile technique throughout. The resulting growth is mainly of diploid cells. To isolate a pure clone of diploid cells, streak out some of the cells from this growth on a fresh plate. Incubate the plate at 30°C for several days until the well isolated colonies are about 0.5 cm in diameter. Then transfer only one-half of a well isolated colony to 0.2 ml of agglutination buffer using a sterile inoculating loop. Divide the cell suspension in half and to each half add 0.1 ml of one of the two testers also in agglutination buffer. If both agglutination assays are negative, then the colony is presumed diploid. The other half of the colony is then picked, purified, and is used to inoculate a stock slant.

Sporulation Assay Grow the diploid strain or a diploid isolated as above in complex medium (M-62) overnight; use this culture to inoculate a slant containing sporulation medium. Incubate the slant for 2 weeks at 30°C. The haploid strains may be treated similarly as controls. To determine if the culture has sporulated, withdraw a loopful of cells and touch to a drop of water on a glass slide to make a dilute suspension. Cover the drop with a cover slip and look for free derby-hat-shaped ascospores, clumps of agglutinated spores, and unruptured asci each containing from one to four ascospores. Sporulation is poor in this strain and never exceeds about 0.1% on this medium. Notice also the production of pseudohyphae on this medium.

Trypsin Digestion The agglutinability of strain 21 is destroyed by trypsin digestion, whereas the agglutinability of strain 5 is unaffected. Cells are prepared for trypsin digestion in the following manner: Resuspend washed cells in 0.01 M tris (hydroxymethylamino methane) buffer at pH 8.5 to a cell concentration of 5×10^8/ml. Withdraw 0.1 ml of each cell suspension (in duplicate for later testing) and to each 0.1-ml sample add 1 ml of trypsin at 250 μg/ml. Prepare the trypsin solution fresh immediately before use by dissolving the weighed trypsin powder (bovine pancreas type 1, 2X crystalized, Sigma Chem. Co.) in a few drops of water first before diluting into the buffer used above. Crude trypsin preparations (e.g., Difco 1:250) can also be used. Incubate the digest for 1 hour at 37°C with occasional mixing and terminate the digestion by centrifuging the cells. Resuspend the cell pellets in 0.1 ml of agglutination buffer and add 0.1 ml of one or the other tester. After digestion, strain 5 should still agglutinate with strain 21 but not with tester strain 5, whereas strain 21 should not agglutinate with either tester.

The agglutinability of strain 5, but not of strain 21, is destroyed by 0.1 M *B*-mercaptoethanol or other reducing agents.

See Wickerham and Burton, 1954; Wickerham, 1956; Brock, 1958a, b, 1961, 1965; Taylor, 1964a, b; Conti and Brock, 1965; Crandall, 1968.

HETEROKARYOSIS IN *ASPERGILLUS*

Heterokaryosis, the intermingling within a single mycelium of unlike nuclei, results from hyphal anastomosis between two unlike parents. Significantly, it is the first event in the "parasexual cycle" that was first demonstrated in *A. nidulans* by Roper in 1952. Heterokaryotic heads developed in crosses between an orange and a brown mutant of *A. carbonarius*, a member of the *A. niger* group, and between the wild type and a white mutant strain of *A. fumigatus* provide easily demonstrated visual evidence of nuclear intermixture. Both crosses may be made on Czapek-steep agar (M-28). Inocula should be prepared as thin-agar suspensions of conidia. Maximum heterokaryon development occurs when conidia of the two mutants are planted as a mixed inoculum. However, a more dramatic demonstration may be achieved when the clones are inoculated singly at diagonal corners of an imaginary 0.5 cm square in the center of the plate to give a culture with four sector-like colonies. Ten days' to two weeks' incubation at room temperature is required.

In cultures of the first type, the resulting single colonies will show abundant heterokaryotic heads intermixed with conidial heads of the two mutants. In cultures of the second type, occasional heterokaryotic heads will develop at the interface between the mutant colonies. In either case, the heterokaryotic heads will be large, black, and indistinguishable from the conidial heads of the parent strain; conidia are multinucleate as they are in the two mutants and in the wild type. Nuclear action within these heterokaryotic heads is "nonautonomous," i.e., the color of a conidium is not controlled by the nuclei within it but by the heterokaryotic conidiophore.

In mixed colonies and at the line of juncture between colonies of *A. fumigatus* and its white mutant, striped heterokaryotic heads composed of some chains of white and other chains of green conidia develop. Conidia are uninucleate and show "autonomous" nuclear action, i.e., the color of a conidium is controlled by its nucleus.

This demonstration may be terminated with an examination of the heterokaryotic heads under low magnification, but is more impressive when dilution platings are made from selected heterokaryotic heads. To accomplish this, conidia are removed carefully from a heterokaryotic head with a very fine needle, suspended in about 0.5 ml of sterile water containing 1:10,000 sodium lauryl sulfonate (wetting agent), stirred vigorously, diluted to a suitable density (1:100 or 1:1T), and 1 ml aliquots plated on Czapek-steep agar according to the customary dilution plating technique. Results of these dilutions can be observed after 1 week (perhaps sooner). Heterokaryotic heads from crosses between *A. carbonarius* mutants will yield pure brown, pure orange, and mixed colonies. Heterokaryotic heads from crosses between *A. fumigatus* stock and its white mutant will yield pure white and pure green colonies but no mixed colonies.

HETEROKARYOSIS AND THE PARASEXUAL CYCLE IN *ASPERGILLUS NIDULANS*

Most stocks of *A. nidulans* held at the Fungal Genetics Stocks Center, Humbolt State College, Arcata, California, derive from those used by Pontecorvo and colleagues. It is to these that the present account refers. Unless the intention is to show heterokaryon incompatibility or other related phenomena, it is important to use strains of common origin.

Stocks may be maintained on complete medium with subculture, by conidia or ascospores, at yearly intervals. Mutation is rarely troublesome except for morphological variants that may arise quite frequently. Purification by single colony isolation is almost invariably sufficient to recover the desired type.

A. nidulans is cultured at 37°C in inverted dishes. For maximum formation of conidia the layer of medium should be rather thin and the dish should not be sealed--this can be avoided by putting a filter paper between the base and lid. On the other hand, to promote formation of perithecia the layer of medium should be thick and, if necessary, dishes should be sealed with adhesive tape.

The symbols of some mutant alleles, and the phenotypes they determine, are listed in Table 11. A lower case initial letter designates a mutant allele recessive to its wild type form in heterozygous diploid; a capital initial designates a dominant or semi-dominant. Wild type alleles are designated by adding "+" to the mutant allele. Genotypes are written as follows: Alleles are given from left to right in each linkage group and the linkage groups, in order from I to VIII, are separated by semicolons. Centromeres are shown by an open circle.

Heterokaryosis

Cultures with a small proportion of heterokaryotic hyphae may be prepared by permitting mixed growth of any two strains. In general it is far more useful to prepare "balanced" heterokaryons in which the hetero-

TABLE 11 Some Mutant Alleles of *A. nidulans*.

Phenotypic Class	Gene Symbol	Phenotype
Conidial color	*y*	yellow conidia
	w	white conidia (*w* is epistatic to color)
Resistance	Acr_1	resistance to acriflavine
	Act_1	resistance to actidione
Colony morphology	*co*	compact, reduced growth rate
	sm	small, reduced growth rate
Nutrition	*paba*	growth requirement for *p*-aminobenzoic acid
	pyro	growth requirement for pyridoxine

karyotic hyphae have a selective advantage over any homokaryotic hyphae. This is most easily achieved through the use of pairs of different nutritional mutants grown on a medium supplying the requirement of neither strain. Alternative balanced systems are discussed later.

A simple and almost unfailing method of heterokaryon systhesis is as follows. A pair of mutant strains is chosen such that the strains differ in their nutritional requirement and in conidial color. A loopful of dry conidia from each strain is floated on the surface of about 2 ml liquid minimal medium (M-83) supplemented with about 1% liquid complete medium. After 2 days' incubation there is a mycelial pad on the surface of the medium. The pad is lifted intact, drained of excess medium on the side of the tube, and transferred to a dish of solid M-83 (a thick layer). The mass of mycelium is teased out into small pieces on the dish and incubated for 3-5 days. The appearance then depends on the particular combination of strains used. Some pieces of teased mycelium may show no growth or very stunted growth. But there are usually one or more sectors of vigorous growth bearing conidia of both colors. Growth within such a sector may be uneven and the conidial ratio, judged visually, may differ substantially from 1:1. Subcultures from the vigorous sectors are made by transferring blocks of agar (1-3 mm cube) with hyphal tips to fresh M-83 dishes. The layer of medium should always be thick so that there is a reservoir for diffusible nutrients produced by the heterokaryon. With two inocula per dish the heterokaryons can be maintained indefinitely with subculture every 3 to 6 days.

Viewed macroscopically, heterokaryons generally appear as a fine mosaic of the two component conidial colors. Microscopically it is usually possible to detect single conidial heads with stripes of color; this is particularly easy with well-contrasted conidial colors such as green and white. The conidia are uninucleate and the "mixed heads" show that determination of conidial color is an autonomous process in which the intermediates are cell-localized. There is an interesting contrast with the situation in *A. niger*, where conidial color determination is nonautonomous. For example, heterokaryotic combination of fawn and olive strains shows a range of conidial colors in different heads; some are fawn or olive, others may approach wild-type color.

Fertile perithecia are usually formed at 8-12 days and are often clustered near the point of subculture.

Colonies of unstable heterokaryons often sector to produce regions of poor growth or regions that appear to carry conidia of one color only. This is particularly so when the heterokaryon is maintained in balance only by certain vitamin requirements. Excess of one nutrient diffuses out into the medium and permits a sector of homokaryotic growth. In stable heterokaryons there is a fine mixture of the two conidial types. There are doubtless some homokaryotic hyphae but the heterokaryotic component has a very great selective advantage. Heterokaryons of the stable type occur when the component strains have requirements for nutrients, such as certain amino acids, that are required in relatively large quantity. Certain mutations determining a reduced linear growth rate (*sm* or *co*) are useful for preparing this class of heterokaryon. For instance, well-balanced heterokaryons can be maintained on complete medium if one component carries *sm* and the other carries *co*.

Adaptation

Adaptation can easily be shown in *A. nidulans* by use of the semi-dominant allele Act_1 that confers resistance to actidione. Heterokaryons of the following types are prepared, though for a simple demonstration either (i) or (ii) would suffice.

(i) $\alpha\ Act_1$ with $\beta\ Act_1^+$
(ii) $\alpha\ Act_1^+$ with $\beta\ Act_1$
(iii) $\alpha\ Act_1$ with $\beta\ Act_1$
(iv) $\alpha\ Act_1^+$ with $\beta\ Act_1^+$

Genotypes differing in genes for nutritional requirement and conidial color are represented by α and β. The heterokaryons are prepared in the normal way and subcultures of all four made to M-83 with 0, 100, 200, 400, 600, and 800 mg/liter actidione. Heterokaryon (iv) is a control that will not grow even on the lowest actidione concentration. Heterokaryon (iii) is also a control, and conidial ratios should show only random fluctuations between different actidione concentrations. However, in the case of (i) and (ii), conidia of the *Act* component increase until, at a certain actidione concentration, a plateau is reached. The plateau presumably reflects the nutritional limits of the particular combination of strains.

If the adaptation is substantial, it can be seen just by inspection of the heterokaryons. Otherwise, it can be demonstrated unequivocally by sampling the conidia widely from each heterokaryon and plating on complete medium. A wetting agent is needed to get even suspension of conidia and a shaker, or Pasteur pipette, is needed to break the conidial chains.

The Sexual Cycle

Since *A. nidulans* is homothallic, heterokaryosis is an essential step in the sexual cycle.

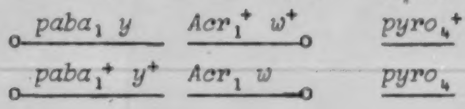
Heterokaryons may yield three classes of perithecia. Two classes contain only "selfed" asci of one or the other parental type. Each of the third class of perithecia, the hybrids, derives from two genotypically different nuclei. A variable proportion of "twin" perithecia may occur; these are perithecia initiated by more than two nuclei. A twin perithecium can have any combination of selfed and hybrid asci.

The frequency of hybrids, which is important when genetic analysis is being undertaken, can be determined as follows. Individual perithecia are picked and rolled over a surface of hard agar (4% in water) to clean them from adhering conidia. Each cleaned perithecium is squashed on the side of a dish of complete medium and the ascospores distributed in a streak towards the center. Eight perithecia can be tested on one dish. After two days' incubation the streaks are inspected. Selfed perithecia yield one color of growth on a streak; hybrids yield the appropriate two or three colors depending on segregating alleles for conidial color. Confusion can arise from contaminating conidia and, more important, from twin perithecia that could in some cases be confused with hybrids.

The Parasexual Cycle

The individual steps in the parasexual cycle are: (1) Heterokaryosis, which brings nuclei of different genotypes into the same cytoplasm; (2) fusion, in the hyphae, of pairs of unlike nuclei; (3) mitotic recombination, a variety of processes in which genes are recombined during vegetative growth. Since certain steps in the parasexual cycle are rare, selective techniques are necessary for their demonstration and effective use. Strains to be "crossed" should differ in nutritional requirements, conidial color where this is possible, and preferably also in other properties such as drug resistance.

Phenomena will be illustrated with the following two strains with centromere positions, as shown:



Diploid Isolation and Recognition. Heterokaryons formed between the above two strains carry only white and yellow conidia. As a very rare event there may be a small or large sector, in the heterokaryon, bearing green conidia that are heterozygous diploids. More commonly, heterozygous diploid conidia constitute only about 1 in 10^6 of all conidia formed by the heterokaryon. These diploids may be selected from the mass of parental haploids by their ability to grow on M-83. A dense suspension of conidia is prepared, as free as possible from hyphal fragments. Up to 10^7 conidia per dish are plated, using a fairly thick layer of M-83. The density of conidia should be regulated so that any slight growth of parental haploids, or reconstituted heterokaryon, does not obscure the diploid colonies. It is usually easy to distinguish diploid colonies from reconstituted heterokaryons that have an irregular growing front. In the present example heterozygous diploids and heterokaryons are readily distinguishable by conidial color. Diploid colonies appear after 2-3 days' incubation and should be transferred by conidia to tubes of M-83 or complete medium. Diploids should not be serially subcultured, since there is a considerable risk of selecting a recombinant type in so doing. The original culture tubes should be used in any series of experiments and a fresh diploid isolated from the heterokaryon when necessary. The following criteria establish diploidy:

- The phenotype. In the present example the diploid would be prototrophic and have green conidia. This is consistent with the reasonable expectation that most mutant alleles would be recessive. The present example carries one of the relatively rare exceptions to this; Acr_1 is semi-dominant.
- The DNA per diploid conidium is, within experimental limits, twice that per haploid conidium.
- Diploid cultures may produce perithecia, but most of these have very few ascospores. Asci may be seen with 16 rather than 8 spores; this does not establish diploidy but is indicative of behavior never observed in haploid strains.
- Diploid strains undergo spontaneous mitotic recombination. In the various segregants and recombinants it is possible, collectively, to recover all the mutant alleles introduced through the parent haploids. Mitotic recombination is treated fully later.
- Conidial size. This is the everyday criterion used to distinguish haploid and diploid strains and results are very rarely ambiguous. Conidia are taken very gently and placed in a drop of an appropriate solution on a slide. A 20% solution of lysol is suitable, but many other fluids can be used. A cover slip is applied without pressure. Chains of conidia are measured with a calibrated eyepiece micrometer; five chains of five conidia each are generally sufficient. Haploid conidia of *A. nidulans* have a mean diameter of about 2.8μ and diploids about 3.7μ. Conidial size is an excellent criterion of ploidy in *A. nidulans* and is usually a good guide in other species. However, difficulties have been encountered in some species, especially those with highly variable or nonspherical conidia. In such

cases a comparison should be made of the mean diameters of each parent haploid and the putative diploid.

Mitotic Recombination The modalities of mitotic recombination will be illustrated by reference to the heterozygous diploid synthesized from the parents shown on p. 501.

There are two processes of mitotic recombination, haploidization and mitotic crossing-over. These processes are entirely independent of each other and any segregant or recombinant will have resulted from one or the other process. The only exceptions are the expected rare types resulting from infrequent coincidence or successive occurrence of the two independent processes.

- Haploidization. This process involves segregation of whole chromosomes without crossing-over, and it is usually a step-wise process. Mitotic nondisjunction, in about 2% of mitoses, gives aneuploids of constitution 2n + 1 and 2n - 1. The latter class generally undergoes successive loss of chromosomes until a stable haploid state is reached. The overall result is the independent segregation of members of each homologous pair of chromosomes so as to give recombination between but not within chromosomes. In the absence of special techniques (see below) the only haploids that would be readily detected are those with white or yellow conidia, and these would occur as sectors or small patches in predominantly green colonies. In the present case all the haploid yellow would require *p*-aminobenzoic acid; about 50% of them would require pyridoxine. The yellow haploids would all be acriflavine-sensitive since they would have to carry w^+ (the allele for color), which is linked in coupling with Acr^+. All the white haploids would be acriflavine-resistant but there would be four nutritional classes depending on the segregation of the chromosomes bearing nutritional markers. Low density platings will often show some of the steps in haploidization. If conidia from a diploid culture are plated at relatively high density, the great majority of resulting colonies carry predominantly diploid conidia, with occasional haploid segregants as patches or sectors. Low density platings reveal a proportion of types that are clearly at a disadvantage in higher density platings. These

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types show centers of stunted growth but produce vigorous sectors; the centers are aneuploid (n + 1, n + 2, n + 3, etc.) and the sectors are usually haploid.

- Mitotic crossing-over. This follows the modalities first worked out by Stern for *Drosophila*. At the four-strand stage of mitosis there are rare but regular exchanges between homologous chromosomes. At any one event there is usually one exchange in one chromosome arm. The subsequent segregation of centromeres is mitotic; if appropriate strands segregate together, the result is a diploid nucleus homozygous for certain markers that were previously heterozygous. In the absence of specialized techniques, mitotic crossing-over is detected only when the products of the process differ visually from the parent diploid. Specifically, mitotic crossing-over, followed by appropriate segregation of strands, brings about homozygosis for all markers linked in coupling and distal to the point of exchange; markers proximal to the exchange and those on other chromosome arms remain heterozygous.

Figure 25 illustrates this with reference just to the right arm of chromosome 1. Consider an exchange as shown in region A followed by segregation to the same pole of strands 1 and 3. The resulting nucleus is homozygous for the markers *paba* and *y*. An exchange as shown in region B, with similar segregation of strands, gives a nucleus homozygous for *y* but still heterozygous for *paba*. Except for extremely rare coincidence of two

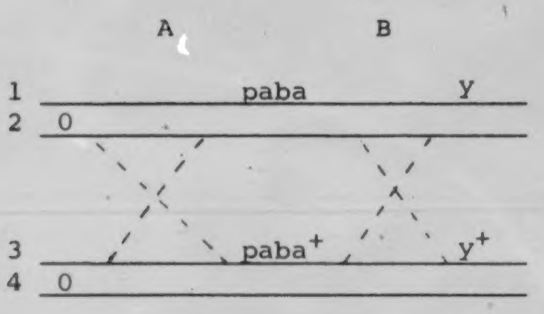

FIGURE 25 *Mitotic Crossing-over in A. nidulans.*

cross-overs in one nucleus, all other markers would remain heterozygous. This could be shown quite readily for the marker *w*, since the yellow diploids should still segregate for white.

Mitotic Analysis

- Visual selection of segregants. This method, the first used and the most laborious, is implied in the above sections. Conidia of a diploid strain are plated at low density (10 or fewer colonies per dish) on complete medium. Yellow or white colonies are ignored, since they represent segregation occurring before plating. Colonies with green conidia are explored under a dissecting microscope at about 10X magnification. Yellow or white segregants are picked with a straight wire and streaked on dishes of complete medium to purify. A maximum of one white and one yellow segregant per colony are picked so as to avoid clones. The dishes are incubated for two days and all subsequent tests made on that part of each streak that is visually pure yellow or white. The segregants are first tested for ploidy. Haploids are then classified for all segregating markers in the way described for meiotic segregants. Deploid segregants are likely to be prototrophic or to require only the nutrients determined by mutant alleles linked in coupling with the color marker. In the present case, apart from rare exceptions, the whites need be tested only on M-83 and the yellows on M-83 and M-83 with *p*-aminobenzoic acid.
- Automatic selection of segregants. A number of methods of automatic selection are available but only one, easy to use and applicable to the present example, is given here. The allele Acr_1 is semi-dominant; haploids of genotype Acr_1 or diploids Acr_1/Acr_1 show a greater linear growth rate, on complete medium with acriflavine, than the heterozygote Acr_1/Acr_1^+. Conidia of the heterozygous diploid are plated on complete medium with 25 mg/liter acriflavine. Depending on both the residual genotype of the strain and the composition of the medium, this amount may need to be varied. Colony density should be kept to not more than five or so colonies per dish. Growth is stunted but after 3-5 days, vigorous sectors (*Acr* or *Acr/Acr*) appear, and

conidia taken from their extreme edges are sufficiently pure for immediate test. In the present case all the haploids would be white; the diploids would be white or green according to the point of cross-over.

- Selection of haploids. The products of haploidization can be selected by the following technique. Conidia of the diploid are plated at low density (5-10 per dish) on complete medium containing 25 mg/liter DL *P*-fluorophenylalanine. The concentration of inhibitor may need adjustment according to the strain used and composition of the medium. Colonies show stunted growth but after 3-5 days vigorous sectors grow out. The sectors are haploids and can be tested immediately for nutritional requirements, etc.

Comparison of Meiotic and Mitotic Recombination Mitotic recombination yields, overall, the same results as meiotic recombination. Mitotic recombination can be used to locate genes and centromeres and to establish gene order within a chromosome arm. The results of such analysis are always in absolute agreement with results via meiosis

Meiotic and mitotic recombination differ in the following particular respects. First, mitotic recombination is a much less efficient process than meiosis for gene reassortment. Second, in meiosis, reduction to the haploid state and crossing-over are, with rare exceptions, part of one cycle of events; in mitosis these processes are independent. Third, it is possible to compare the relative frequencies of crossing-over, at meiosis and mitosis, for particular regions of the linkage groups; the two cross-over processes show different patterns of frequency distributions.

See Pontecorvo *et al.*, 1953a, b; Pontecorvo and Sermonti, 1954; Käfer, 1958, 1962; Pontecorvo and Käfer, 1958; Warr and Roper, 1965; Clutterbuck and Roper, 1966; Roper, 1966.

GENETIC STUDIES WITH *USTILAGO MAYDIS*

U. maydis was originally selected for genetic studies because it has a much shorter life cycle than most other species. When young seedlings are inoculated with haploids of opposite mating type, the infective heterokaryon or dikaryon causes the growth of white swellings or galls on the leaves, leaf bases, and stem after 4-6 days. Smut spores (teliospores) are formed within the galls in very large numbers 8-10 days after inoculation.

In *U. maydis* sporidia do not fuse in pairs to form heterokaryotic cells. Although a linear tetrad of primary sporidia is sometimes formed, more often the pattern of sporidium formation is irregular. The simplest type of genetic analysis involves scoring the random products of meiosis. Attempts have been made to complete the life cycle in artificial media, but so far teliospores can only be formed on the host.

U. maydis is heterothallic; the details of the mating system were first worked out by Rowell and DeVay (1954). Fertility between haploid strains depends on two genes, the *a* gene has two alleles and the *b* gene has multiple alleles. Cells with different *a* alleles will fuse under appropriate conditions to form a heterokaryon, which will only infect the host and allow the completion of the life cycle if the *b* alleles are also different. The *a* gene can, therefore, be regarded as the mating type locus that is homologous to the simple two-allele mating system of most smut fungi, whereas the *b* gene can be regarded as one controlling pathogenicity. In *U. maydis* it is possible to obtain vegetative strains that are diploid and heterozygous for the mating type and pathogenicity genes (Christensen, 1931; Holliday, 1961a, b; Puhalla, 1969); they are referred to as solopathogenic strains since they will infect the host when inoculated alone into it.

Media

U. maydis is undemanding in its nutritional requirements and will grow on standard synthetic minimal media such as Czapek Dox, or the Vogel and Bonner *Neurospora* Minimal medium. Such media can be enriched with yeast extract, malt extract, hydrolyzed casein or bactopeptone in order to provide a simple complete medium. Media containing too high a concentration of bactopeptone or hydrolyzed casein, while allowing vigorous growth, will result in the rapid death of most of the cells after growth has ceased. Again, Czapek Dox liquid medium has been found to give poorer growth than the same agar medium unless trace elements, including calcium, have been added.

The media that have been routinely used in Holliday's laboratory are as follows: minimal (M-100-a); complete (M-100-b); salt solution (M-100-c); trace element solution (M-100-d); vitamin solution (M-100-e); nucleic acid hydrolysate (M-100-f).

Individual growth factors are added to minimal medium as required; amino acids, 100 mg; purines and pyrimidines, 10 mg; and vitamins at the concentration given, per liter. In order to simplify the complete medium, the vitamin solution and the hydrolyzed nucleic acid could be omitted and the yeast extract increased from 0.1% to 1%.

Growth

Cultures are incubated at 30°C. For growth in liquid media a mechanical shaker is essential. In liquid complete medium, cells divide every 2 hours and the final stationary phase population will be about 10^8 cells/ml. In liquid minimal medium, cells divide approximately every 2-1/2 hours and the final population is about $5x10^7$ cells/ml. On complete agar medium, colonies from single cells reach 2-3 mm in diameter after 3 days' incubation, which is the appropriate size for replica plating; counting can usually be done after 2 days' incubation. Replicas on minimal medium are always incubated 2 days before examination. In general, streaks for providing cells for inoculating into maize, auxanographic tests, etc., are incubated for 2 days. Cells growing on complete agar can be suspended in water by vigorous shaking, but those grown on minimal medium will not form a suspension of single cells, unless Czapek Dox is used.

Counting and Plating

Cells can be counted with a haemocytometer, or better, with a Coulter electronic particle counter, if one is available. Dilutions are made in sterile distilled water; saline is unnecessary. Although viable cells can be counted by spreading on the surface of agar plates, larger numbers of colonies can be accommodated in a plate if the cells are embedded in molten agar at 45°C. The colonies remain small and this facilitates counting. Cells can be easily washed by centrifuging and resuspending. The cells are somewhat less dense than those of yeast, but centrifuging speeds can be low enough to use standard test tubes both for growing the cells in the shaker and for spinning them down.

Replica Plating

It is important to use good-quality velvet with a dense pile. This will stand up to repeated washing and sterilization in the autoclave as well as providing an excellent inoculating surface. The pieces of velvet should be about 15 cm^2; they can be used damp (after autoclaving) or dry. A piece is spread over an inverted cork or rubber stopper (8 cm in diameter and fixed on an appropriate stand) and held in place with a wire loop. Press the master plate firmly onto the velvet surface and then, without delay, the plate to be inoculated. Normally, the inoculum from each colony is invisible, but occasionally a few of the colonies are removed from the master plate by the velvet. When this happens, the colonies still produce a normal inoculum on the replica, and their position on the master is established by allowing further growth of the few cells that have not been removed. Good illumination is essential when matching up the replica with the master plate. Having located gaps in the replica, it is advisable to examine these with a dissecting microscope to check that cells have been transferred and are in fact not growing.

Preservation of Stocks

U. maydis will not remain viable on agar slants for long periods. Stocks should be preserved in anhydrous silica gel. The gel should be in the form of fine granules, not large pieces; that used for chromatographic adsorption is ideal. Small test tubes containing 2-3 ml silica gel are sterilized and desiccated by heating in an oven. When cool, the tubes are sealed with rubber plugs previously sterilized in the autoclave. Alternatively, small screw-cap vials can be used, provided the caps provide an airtight seal. To preserve a culture, take a large number of cells from a streak on complete medium and transfer to a tube of silica gel. This can be done with a loop or a sterile flat toothpick--the volume of cells should be 10-20 mm^3. The cells are dehydrated very quickly by the silica gel; they retain their viability just as they would after the more elaborate procedure of freeze drying. In order to recover viable cells from a silica gel culture, the lump of dried cells is first broken into small fragments with a sterile metal rod; then a few of these fragments, together with granules of silica gel, are tipped onto a plate or slant. The culture is then sealed until it is required again. It is not necessary to use silica gel when colonies or streaks need to be kept viable for only a few weeks. Viability will be maintained for this period at any temperature between 5° and 20°C.

Mating Type Tests and Genetic Crosses

Mating type compatibility can be determined either by a modified Bauch test in which production of aerial dikaryotic infection hyphae can be seen on agar medium, or by infection of the host.

The first method employs a supplemented agar medium. This may be double strength complete medium in which all components of M-100b except agar and distilled water are doubled (Puhalla, 1968), liquid complete medium supplemented with 1.7% Difco cornmeal agar (Holliday and Resnick, 1969), or minimal medium (M-100a) supplemented with 1% activated charcoal (Day and Anagnostakis, 1971a). Fresh, day-old cultures on complete medium are used and the cells are applied to the mating agar with a sterile toothpick or, as a suspension, with a loop or Pasteur pipette. The strains to be mated should be placed no more than 1 mm apart. A 10-cm Petri dish will conveniently accommodate 16 or 25 unknowns each surrounded by 4 testers. The plates are sealed with adhesive paper tape and incubated at 25°C in continuous light. They are scored after 2 days with a dissecting microscope and side lighting. The dikaryotic hyphae appear as a band of white mycelium only at the junction of compatible colonies. Colonies with mycelial, ropy or flaky morphology sometimes do not show mating reactions on agar.

Diploid colonies heterozygous for *a* and *b* are covered uniformly with mycelium under the test conditions. When whole tetrad colonies from germinated teliospores are grown under these conditions, they each appear as a mosaic of yeastlike cells and mycelium. Unreduced diploid cells from teliospores in which meiosis has failed form mycelial colonies and can be readily detected (Day and Anagnostakis, 1971b).

Mating type determination may also be assisted by making use of the marker pan_1 (requirement for pantothenate), which is very tightly linked to the *a* locus. If pan_1 is introduced into the cross, the *a* mating type of the pan_1 and pan_1^+ progeny is almost always the same as the parents.

When test mating on agar is unsatisfactory or when genetic crosses are to be made, the method of host inoculation is employed. Seeds of a susceptible variety of maize, such as Golden Bantam, are germinated on moist absorbent cotton in metal trays at 25°-30°C. It has been found that the seedlings often get heavily contaminated with fungi, particularly *Rhizopus*. In order to avoid this, the seeds are soaked for 30 min in a solution of 0.1% mercuric chloride containing a wetting agent. After rinsing with sterile water, they are spread on the cotton, which has been previously sterilized and covered with another layer of moist sterile cotton. The tray is then covered with a lid to conserve moisture and sterility. After 3 or 4 days' incubation, the seedlings with a coleoptile 1/2" to 1-1/2" in length are suitable for inoculation; 4-6 seedlings are inoculated for a mating type test; 8-12 for making a cross. The seedlings are placed in small pots half filled with vermiculite moistened with Hoagland's solution {$Ca(NO_3)_2$, 0.82%; KNO_3, 0.51%; $MgSO_4$, 0.25%; KH_2PO_4, 0.14%; ferrous sulphate, 0.01%}, and then covered with vermiculite. The seedlings are incubated at 25°-30°C under illumination, and the humidity should be high. There are three methods of seedling inoculation available:

- Cells from fresh streaks on complete medium or from liquid cultures are mixed together in roughly equal concentrations in water to give a suspension of about 10^7 cells/ml. This can be conveniently done in small screw-cap bottles. The tip of the coleoptile of the seedling is removed and the suspension injected just above the first node with a hypodermic needle until a drop of the suspension emerges from the cut end. Syringes should be 1-ml capacity; they can be sterilized before a further inoculation by rinsing in alcohol, followed by sterile water.
- A quicker and simpler method that is somewhat less reliable than the above is to cut off the coleoptile 2-3 mm above the node and jab the exposed end successively into streaks on agar of the strains to be tested or crossed. Most of the seedlings easily survive this drastic treatment and galls are produced at about the same time as with other methods of inoculation.
- A partial vacuum method of inoculation has been devised by Rowell and DeVay (1953). Tubes about 1" in diameter with a side arm (or filter flasks) are required. The coleoptile tips are removed; the seedlings are placed in the tube and covered with the sporidial suspension. The tube is closed with a rubber stopper and the tube partially evacuated from the side arm (approximately 30 cm mercury). Air is withdrawn from the seedlings and when the vacuum is released, cells are drawn into the spaces previously containing air. This method is very useful for inoculating large numbers of seedlings quickly, but it has no advantages over the other methods for genetic studies.

It may be more convenient to inoculate larger potted plants in a growth room or greenhouse at 25°-30°C. Plants 12"-18" high are inoculated with a sporidial suspension, by means of a syringe, at several points above the first node. Two or three plants are adequate for each test or cross. The galls and spores appear later than when seedlings are inoculated, but the final yield of teliospores is of course much greater. This may be an advantage where a cross is being analyzed, particularly if many students are involved in the exercise.

Teliospores should not be harvested as soon as they become visible in the galls, since they are then immature and germinate poorly. Spores in seedlings should be left in the galls at least 2 days after they first appear or until the seedlings begin to wilt. Gall tissue is crushed in a mortar; 1.5% copper sulphate solution is added and the suspension of spores and debris filtered through moist absorbent cotton in a thistle funnel. The spore suspension is left 24 hours or overnight, during which time contaminants and vegetative cells are killed; it is then centrifuged, the spores washed in water and finally resuspended in a small volume of water. Spores are spread on plates of complete agar and incubated. If bacterial contamination is experienced at this stage, 0.05 ml of a 0.1% solution of achromycin (tetracycline hydrochloride) can be spread on each plate with the spores. Spores germinate after about 18 hours, but the percentage germination varies from cross to cross; so too does the synchrony of germination and the appearance of the tetrad of primary sporidia. The analysis of the products of meiosis will be dealt with in a later section.

Isolation of Biochemical Mutants

Wild-type sporidia of *U. maydis* require no organic growth factors, and there is no difficulty in isolating mutants that require a particular vitamin, amino acid, pyrimidine, purine or reduced sulphur or nitrogen. Such mutants were first isolated by Perkins (1949), using a method that has been found to be less successful than those to be described here. Biochemical mutants, or auxotrophic mutants, are of course invaluable in genetic studies. They segregate cleanly and are easily scored. Morphological mutants are common in *U. maydis*, as was shown by Stakman and his co-workers (Stakman *et al.*, 1943), but they have not been found to be particularly useful as genetic markers, mainly because they are affected by modifier genes and therefore do not segregate cleanly. Two methods for obtaining biochemical mutants have been used: The first involves the use of a mutagen and is non-selective; the second involves selection and mutagenic treatment can be dispensed with. Both methods depend on the technique of replica plating with velvet.

Induced Mutation Ultraviolet light from a germicidal low-pressure mercury lamp is a convenient mutagen. Before attempting mutation experiments, a survival curve should be obtained. The standard method for doing this experiment is to irradiate a low concentration of haploid sporidia in a water suspension with continuous agitation; 1-ml samples are pipetted into Petri dishes (two or three plates for each dose), molten agar at 42°-45°C is poured on and the cells evenly dispersed by mixing before the agar sets. Plates are incubated 3-4 days before counting. With ultraviolet light the yield of mutants does not increase linearly with dose; a dose that kills 90% of the cells will give as high a yield of mutants amongst the survivors as one killing 99.9%. Therefore the survival curve need not be plotted for high doses; if the original suspension contains 500 cells/ml, the dose giving 10% survival will be easily measurable. In order to get reproducible results it is necessary to use cells in the same physiological condition. Cells in log phase in liquid culture are more sensitive to radiation than cells that have reached stationary phase. Either population can be used, but cells should not be harvested from streaks on plates, since the population will consist of a mixture of growing and nongrowing cells. Another method for obtaining survival curves that has been used very successfully is simply to streak the cells at low concentration on agar plates, irradiate the plates with different doses and then score, by microscopic examination, growing and nongrowing cells after 24 hours' incubation. In fact, it is possible by shielding parts of the open plate to measure the killing effect of more than one dose on a single plate. The method, therefore, has the virtue of speed as well as economy in terms of plates, media, and pipettes. It is, of course, unsuitable for obtaining survival curves that include readings for the doses that kill a very high fraction of the cells.

For a mutation experiment, cells are irradiated in water at a concentration such that 0.1 ml will contain about 200 surviving cells. Aliquots of this volume are spread on dry plates of complete medium and incubated for 3 or 4 days, i.e., until the colonies are 2-3 mm in diameter. The plates are then replicated to nitrate minimal medium. In *U. maydis* the fraction of survivors that is biochemically deficient is rather low, usually from 0.2-0.3%. For the most part, colonies that grow slowly on minimal medium should be ignored, since it has been found that they usually do not respond clearly to any growth factor and are useless as genetic markers. However, a particular class of mutants that is quite common (approximately 25% of all auxotrophs) produces a characteristic thin growth on minimal medium. These are mutants that will not use nitrate as a sole source of nitrogen; they will not grow in nitrate liquid minimal medium and their slow growth on the same solid medium is due to small amounts of reduced nitrogen in the agar. Some of these mutants will grow on nitrite; they lack nitrate reductase activity. Others will not grow on nitrite but will accumulate it if given nitrate; these have nitrate reductase activity but lack nitrite reductase.

Selection In order to collect large numbers of mutants it is desirable to have a selective method. This has been achieved by using an inositol-requiring mutant obtained by induced mutation. In several fungi it has been found that such mutants die very quickly when deprived of inositol; this is believed to be due to unbalanced growth connected with the specific failure to synthesize cell membranes. If another metabolic block is introduced into the cell, for instance by a second biochemical mutation, then the unbalanced growth does not take place, or is less extreme, and the cells die much more slowly. This means that if a large population of inositol-requiring cells is plated on minimal medium, after incubation for 2-4 days the vast majority of cells are dead, but any doubly auxotrophic cells have a selective advantage and may be alive at the end of the period of inositol starvation. The selection is in fact so strong that it is unnecessary to use a mutagen to increase the frequency of auxotrophs in the population.

The simplest procedure has been found to be also the most effective. Cells from a fresh streak on complete medium of the haploid strain *inos*$_3$ are harvested on the end of a spatula. The cells are spread with the spatula on one or two plates of complete medium (approximately 10^7 cells/plate), which are incubated overnight. The fresh growth next day is used as the inoculum for spreading cells on plates of minimal medium. Again, a spatula can be used to collect the inoculum and for spreading, but in this case approximately 10^8 cells should be spread per plate. This apparently crude method has the advantage that each inoculum for each minimal plate can be taken from a different part of the complete plate, thus mutants isolated on different plates are almost certain to be of separate origin, whereas those on the same plate may be clonally related. The minimal plates are usually incubated for 3 days, although incubation for 2 or 4 days has also resulted in good mutant yields. After this time the whole piece of agar in each plate is lifted out with a wide spatula and placed on a plate of complete medium supplemented with 10 µg/ml of inositol. On further incubation, the survivors of inositol starvation grow up to form colonies. These are now replicated to minimal medium supplemented with inositol. The fraction of new auxotrophs among the survivors of inositol starvation varies from a few percent to over fifty percent; the method is therefore a highly efficient one for obtaining such mutants. However, it suffers from the disadvantage that often a high fraction of mutants on one plate are identical, being the descendants of a single mutation that occurred during the growth of the inoculum on complete medium. In addition, the range of mutants that are selected tends to be more limited than with the induced mutation method; apart from requirement for nicotinic acid (niacin), vitamin mutants are rather uncommon.

Identification of Biochemical Requirements

If the requirements of only a few mutants are to be identified, auxanographic techniques are most convenient. A plate of minimal medium (or this medium plus inositol) is divided into four sectors by cutting out two strips of agar with a scalpel. Inoculate each sector at a premarked point with each mutant to be tested, up to 12 per sector. (Convenient inoculating instruments are sterile flat toothpicks or three tungsten or nickel chrome wires twisted tightly together; sufficient inoculum can be held on the end such that each sector can be touched in turn without replenishing the inoculum.) Supplement two of the sectors with a small drop of vitamin solution and hydrolyzed nucleic acid solution (those used in the complete medium) and the other two with a small but visible amount of hydrolyzed casein and ammonium sulphate. Incubate 2 days. This initial classification is followed by testing the growth response of mutants to individual amino acids, purines, pyrimidines and vitamins, as well as such inorganic supplements as nitrite, sulphite, thiosulphate (reduced sulphur mutants will have grown on hydrolyzed casein, since they will respond to methionine and cysteine). Up to eight or ten sectors can be cut in a plate, and the supplements added as crystals taken directly from the bottle with a moist wire or loop. Finally, unequivocal identification can be carried out by streaking the mutant across a plate of minimal medium and spotting with the growth factor at one side; a graded growth response to the supplement will be observed. When the requirements of a large number of mutants are to be identified, it is easier to replicate these from a master plate to various test media (Holliday, 1956) than to use auxanograms. The commoner categories of mutant are: Requirement for arginine (or ornithine), leucine, lysine, methionine (or cysteine), adenine, cytidine or uridine, nicotinic acid, pantothenic acid choline, thiamin, pyridoxin, inositol, *P*-aminobenzoic acid, reduced sulphur (sulphite or thiosulphate) and reduced nitrogen (nitrite or ammonia).

Temperature-Sensitive Mutants

Any gene that specifies the structure of a protein can probably mutate to a form that results in the production of a protein that is unstable or functionless at high temperature, whilst retaining its normal function at a low one. These mutants are of particular importance in genetic and physiological studies, since they allow one to obtain mutations in a large number of indispensable genes. The same replica-plating technique that is used to obtain auxotrophs can be used to obtain temperature-sensitive mutants; indeed both kinds of mutant can be isolated from one experiment. The UV-treated cells are incubated at a low temperature, say 20°C, and the colonies replicated and incubated at 30°-32°C. The proportion of temperature-sensitive mutants that is obtained is similar to that of auxotrophs.

Random Products of Meiosis

The presence or absence of meiotic linkage between genetic markers can be most readily examined by analysis of the random products of meiosis, rather than by tetrad analysis, although the latter is entirely feasible. The method that has been used is possible only because of the failure of *U. maydis* to form heterokaryons readily on most agar media. A large number of teliospores from a cross between auxotrophic markers are germinated on complete medium. When individual colonies are barely visible to the naked eye, 5-10 ml of sterile water is added to the plate and the cells rubbed off the agar with a spreader or spatula. The suspension, from one or several plates, is pipetted into a screw-cap bottle and vigorously shaken to form a suspension of haploid cells. This is then counted, diluted and plated at the rate of about 100 cells/plate of complete medium. The colonies are replicated to minimal medium supplemented to form a single omission series; e.g., if auxotrophic markers *a*, *b*, and *c* are segregating, then all the eight possible genotypes are identified by replicating to media containing requirements *ab*, *bc*, and *ac*. It is usually advisable to have a test plate containing all the relevant supplements. To do this makes it easier to decide whether a growth response on one of the other plates is positive or negative. The scoring is done by numbering at random all the isolated colonies on one or more of the complete plates and subsequently scoring their requirements, and therefore their genotype, by examination of the test plates. This can be most easily done by scoring the responses of all the colonies on a plate to each test medium by matching up each replica in turn, rather than by matching up each replica for each colony in turn.

It is instructive to follow the segregation of the markers *na* and *ni*, since the position of the metabolic block is known in each case; *na* mutants lack nitrate reductase--they will grow on nitrite (0.03% in minimal medium) but not on nitrate as sole nitrogen source; *ni* mutants lack nitrite reductase--they cannot grow on nitrite or nitrate, but if given nitrate, they convert it to nitrite, which accumulates in large quantities in the medium. When both markers are segregating, it is necessary to examine nitrite accumulation in order to score the four possible genotypes. This is shown in Table 12.

Colonies that do not grow on nitrite are transferred to tubes of solid or liquid nitrate medium, supplemented as necessary if other markers are segregating in the

TABLE 12 Segregation of Genes Controlling the Reduction of Nitrate.

Genotype	Growth on			Accumulation of Nitrite on Nitrate Medium
	NO_3	NO_2	NH_3	
na^+ ni^+	+	+	+	(slight)
na ni^+	-	+	+	-
na^+ *ni*	-	-	+	+
na ni	-	-	+	-

cross. After 24 hours' incubation, the presence of nitrite is tested by adding 1 ml of a solution of 1% sulphanilamide in 1 N HCl and 1 ml of a solution of 0.02% N-1-naphthyl ethylene diamine dihydrochloride. If the genotype is *ni*, the sulphanilamide is diazotized by nitrous acid and couples with the amine to form a deep pink color, whereas *na ni* isolates produce no color. For comparison, a wild type and an *na* isolate should also be tested in this way.

The use of this method of genetic analysis is based on the assumption that the growth rate of the primary products of meiosis is independent of genotype. To a large extent this is true, since in many crosses, including some with up to eight markers, there is a 1:1 Mendelian ratio for each marker, and complementary genotypes are equal in frequency. In these cases the frequency of recombinant genotypes is a valid measure of the linkage, if any, between genes. In some crosses, however, particular auxotrophic markers clearly restrict growth rate or basidiospore germination, since the wild type allele is significantly more frequent. These crosses will, nevertheless, often make it possible to recognize the existence of linkage, although not to measure it with accuracy. Extensive mapping has not been carried out with *Ustilago*; by random spore and tetrad analysis, linkages involving about 12 markers have been established.

Mitotic Crossing-over in Vegetative Diploids

Long before Pontecorvo and his associates discovered vegetative diploid strains in *Aspergillus nidulans* and related species, Christensen had identified them in *U. maydis* (Christensen, 1931). He was able to recognize such strains by the fact that the sporidia were uninucleate and at the same time homothallic or solopathogenic. They arose as a result of failure of reduction of chromosome number at meiosis in certain crosses. When inoculated into the host, they produced galls and teliospores. In many cases when these were germinated, segregation of the mating type factors occurred, thus showing that the solopathogenic strain was heterozygous for such factors.

Since the origin of diploids at meiosis is sporadic, more reliable methods for obtaining them are routinely used. Different auxotrophs of opposite mating type are inoculated into maize seedlings. When the galls have appeared, but before the appearance of teliospores, pieces of infected tissue (about 10 mm^3) are cut out from the center of a gall with a sterile scalpel and placed on minimal medium, three to five pieces per plate. If the galls are very small, whole galls can be surface-sterilized with hypochlorite solution, which contains a wetting agent, and then rinsed before plating on minimal medium. After several days' incubation, small colonies will invariably grow out from the pieces of tissue. These are vegetative diploids heterozygous for the recessive markers originally present in the haploids. Their selection is possible since neither haploid cell would grow on minimal medium, nor will the dikaryon, since it is an obligate parasitic stage of the life cycle. Presumably the diploids arise from cells that would normally give rise to teliospores.

An alternative, but quite similar, method (Puhalla, 1969) for synthesizing diploids makes use of minimal medium instead of host tissue. This method has the added advantage that it also works with haploids having the same *b* factor and so can be used to recover diploids that are homozygous for *b*.

The diploidy of the prototrophic colonies obtained by these methods has been proven by measuring their DNA content compared to haploids and also by observing their genetic behavior. The diploids heterozygous for *a* and *b* are homothallic, since if inoculated alone into maize, teliospores are produced. At meiosis, the markers that were originally present in the haploid parents show normal segregation. The diploid strains are stable, but they do show rare spontaneous somatic segregation. This can be detected by plating diploid cells on complete medium and replicating to minimal medium. About 1/1000 of the colonies is auxotrophic, but the exact frequency depends on the number of markers in the diploid. The frequency of somatic segregation can be greatly increased by treating the diploid cells with UV light. A dose that kills 90% of the cells will increase the spontaneous frequency 10-20 times, and higher doses have a greater effect (unlike the situation with regard to

UV-induced mutation). Both the spontaneous and the induced segregants are diploid, not haploid, and they arise as a result of the process of mitotic crossing-over. This process, which is illustrated in Figure 26, results in homozygosity from the point of the exchange to the end of the chromosome arm, in half the cases. Mitotic crossing-over makes it possible to assign markers to different chromosome arms and to determine the order of markers within arms. Excellent reviews of genetic analysis by means of mitotic crossing-over are available (Pontecorvo, 1958; Pritchard, 1963). In *U. maydis* such analysis has confirmed linkages detected by meiotic analysis and has shown that there are at least five chromosome arms, thus showing that the cytological count of two chromosomes in haploids is an underestimate, and casting doubt on the same count in other species.

It is frequently found that segregant colonies are mosaic, consisting of the reciprocal products of the exchange. If such mosaics are half prototrophic and half auxotrophic, they are detected directly as semicircular replicas on the minimal plate. Mosaics that are wholly auxotrophic are obtained only when each parent haploid carries a marker in the same chromosome arm. A cross-over between the centromere and the nearest (proximal) marker will in half the cases produce one daughter cell homozygous for one marker and one homozygous for the other. Such mosaics are recognized by the appearance either of the colony on complete medium or of the cells on the replica; otherwise they are

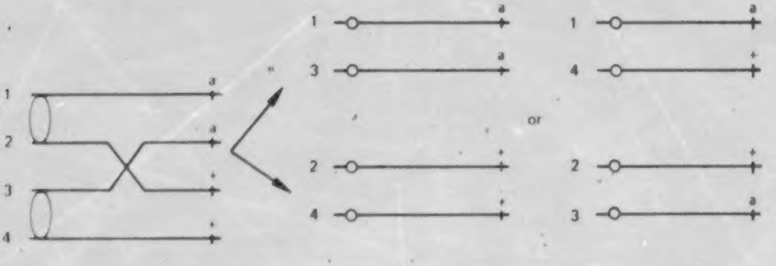

FIGURE 26 *The genetic consequences of mitotic crossing-over.*

detected as mixed phenotypes during the identification of the requirements of the segregants. After irradiation it is not surprising that many recombinant colonies are not mosaic, since it would be expected that frequently only one of the two daughter cells would survive. The various types of recombinant that can be detected from a diploid heterozygous for three markers on the two arms of one chromosome are shown in Table 13. The phenotypes of the segregants are usually identified by removing cells from the replica on small pieces of agar cut out with a sharp needle under the dissecting microscope and transferring them to a plate of complete medium (but if the *inos* is segregating from a diploid, the cells on minimal may be dead). The master plate is then replicated to appropriate test media. Since double crossovers on different arms are uncommon, the majority of the segregants will have a single requirement (but see Table 13); most of the test media should therefore have single supplements, as this will facilitate the identification of wholly auxotrophic mosaics. (Any recombinants that do not grow on these media can be tested later by auxanography.) If a single omission series of test media was used, as in meiotic analysis, then auxotrophic mosaics would grow on all the media.

Apart from prototrophic diploids, it is also possible

TABLE 13 Phenotypes of Recombinant Colonies (see Holliday, 1961b).

		Position of Cross-over			
	Genotype of Diploid	Single Exchange			Double Exchange
Marked interval:	I II III	I	II	III	I & II
Example 1	$\frac{a\quad b\quad\circ\quad c}{a^+\ \ b^+\qquad\ \ c^+}$	a or $a/+$*	ab or $ab/+$	c or $c/+$	b or $b/+$
Example 2	$\frac{a\quad b^+\quad\circ\quad c^+}{a^+\ \ b\qquad\ \ c^+}$	a or $a/+$	a, b or a/b	c or $c/+$	$b/+$

*The survival of reciprocal products of mosaics, e.g., $\frac{a\ b\ c}{a\ b^+c^+}$ with $\frac{a^+b\ c}{a^+b^+c^+}$, represented here by the phenotype $a/+$.

to synthesize heteroallelic diploids, i.e., diploids that contain in the trans configuration two noncomplementing mutants in the same gene. Such diploids were first studied in yeast and *Aspergillus*. In *Ustilago* the diploids can be obtained only if the mutants are fertile when crossed, as is often the case. The haploids carrying distinct mutants of the same gene must also have different auxotrophic marker(s), so that the diploid can be selected in the usual way on a medium containing a single supplement. Heteroallelic diploids will revert to the wild-type phenotype following intragenic recombination. Such recombinants can, of course, be selected from a large population of nongrowing cells on minimal medium. Very low doses of UV light enormously increase the frequency of recombinants, much more so than it increases the rate of back mutation in haploid or homoallelic diploid mutant strains. It is believed that the effect of UV light in stimulating both intergenic and intragenic recombination is due to induced pairing of synapsis of homologous parts of the genome (Holliday, 1964). Studies on the mechanism of recombination are being continued with heteroallelic and heterozygous diploids of *Ustilago*.

See Rowell, 1955; Holliday, 1961b, 1962, 1964; Esposito and Holliday, 1964; Puhalla, 1970; Anagnostakis, 1971; Day *et al.*, 1971; Hankin and Puhalla, 1971.

HETEROTHALLISM IN BASIDIOMYCETES

Heterothallism among the higher Basidiomycetes involves no morphological differentiation, but only genetically controlled physiological differentiation. Heterothallic Basidiomycetes are of two basic types: *Bipolar*, in which mating competence is determined exclusively by the segregation of alternate incompatibility factors of one series; *tetrapolar*, in which mating competence is determined by the segregation and assortment of incompatibility factors of two series. In both types of heterothallism, a large number of alternate and equivalent factors has been found to occur in each series.

Fertile sexual interactions are possible only between haploid mycelia that are compatible, that is, carry different factors of each series, thus:

Bipolar	- A_1 X A_2	dikaryon	completion of life cycle
	A_1 X A_3	"	" " " "
Tetrapolar	- A_1B_1 X A_2B_2	"	" " " "
	A_1B_1 X A_6B_4	"	" " " "

There is only one class of incompatible interaction in bipolar species, while there are three in tetrapolar species. These incompatible interactions, and the resulting heterokaryons are as follows:

Bipolar	- A_1 X A_1	Common-factor heterokaryon
Tetrapolar	- A_1B_1 X A_1B_2	Common-*A* heterokaryon
	A_1B_1 X A_2B_1	Common-*B* "
	A_1B_1 X A_1B_1	Common-*AB* "

Each of these heterokaryons, as well as the dikaryon, possesses a distinctive phenotype that can be used to distinguish among the different mycelial interactions that may occur in a given species. These phenotypes are described below in the context of the descriptions of the mating reactions.

Collection and Storage of Materials

Fruiting bodies (sporocarps) of *Polyporus betulinus* (a bipolar species) and *Schizophyllum commune* (a tetrapolar species) are widely distributed and are extremely characteristic, so that suitable specimens may be obtained and identified without difficulty.

P. betulinus appears to be restricted to moribund and decaying *Betula papyrifera* (white- or paper-birch), on which it forms conspicuous fruiting bodies. Nearly every unpruned stand of birch will provide suitable fruiting bodies. Avoid dried or discolored material in favor of tan specimens with an exposed whitish porous undersurface. Specimens may be collected during early to mid-Fall, preferably after the first or second frost. Fruiting bodies should be placed in individual plastic or paper bags and should be kept cool (but not refrigerated) until needed.

S. commune is world-wide in distribution, and fruiting bodies may be found on practically all woody plants; fallen twigs, branches, stumps, logs, etc., are usually better sources than healthy, intact trees. Collections may be made throughout the year, and specimens may be air-dried and stored in paper boxes or envelopes until needed.

The following media and techniques are routinely employed:

- For *S. commune*, complete (CM) (M-98b); minimum (MM) (M-98a); complete plus yeast (CYM) (M-98c). These are used as follows: Isolation of monosporous progeny (CM or CYM); vegetative cultures (MM, CM, or CYM); matings (for study) (MM or CM); fruiting of dikaryons (MM or CYM); and storage (CYM).
- For *P. betulinus*. While a simple minimal medium has not been developed for *P. betulinus*, *Schizophyllum* CYM is suitable for most applications. MEM Difco Malt Extract (2% W/V) solidified with agar (2% W/V) supports more vigorous vegetative growth and more rapid fruiting. (Note: Difco Malt Extract Agar is a *different* medium from the one suggested and is *not* generally suitable.)

Stock collections may be maintained at 5°C on CYM slants in tightly closed, screw-capped vials or small bottles (e.g., Duraglas A4350). Standard screw-capped test tubes are too deep for the convenient retrieval of the culture and are thus unsuitable. Cultures of *P. betulinus* maintained under these conditions remain viable for 6-7 months, while similar cultures of *S. commune* remain viable for 24-36 months. The useful life of a stock culture may be greatly extended by completely filling the vial or bottle with sterile, light mineral oil. CYM slants of *S. commune* have been stored at 20°C for up to 5 years without loss of viability. Since some random attrition is unavoidable under each of the three storage conditions specified, it is advisable to maintain duplicate sets of stock cultures at 5° and at -20°C.

The accumulation of spontaneous mutations during storage represents the major difficulty in the long-term maintenance of culture collections. Cultures of *S. commune* (and probably of *P. betulinus*) accumulate spontaneous morphological and other mutations during prolonged vegetative growth and even during extended storage in the cold. Many of these mutations render stocks unilateral, i.e., unable to accept nuclei in matings. It is best to collect fresh material from nature whenever possible rather than rely on stock cultures.

Isolation of Homokaryotic Strains

Homokaryotic strains of both species may be obtained by isolating individual germlings from agar plates on which basidiospores were spread and allowed to germinate. Basidiospores should be collected from fruiting bodies of *P. betulinus* during the 24-36 hours following collection; fruiting bodies of *S. commune* may be used either fresh or for up to five years if dried and stored. Excise a 1/2 cm^2 of fruiting surface (or use an entire fruiting body of *S. commune*) and attach this to the inside of a Petri dish lid with Vaspar or vaseline, so that the fruiting surface is parallel to the surface of the lid. Place the lid over a plate of MEM or CYM and mark the position of the sporulating tissue on the lower surface of the plate with a glass-marking pencil. The density of the spore deposit can be determined by examination with the low-power objective of a compound microscope. Either with fresh materials or with dried material that has been wetted, it is impossible to predict either the delay in the initiation of basidiospore discharge or the rate of spore deposition once begun. A practical solution is to examine the spore deposit at 1-2 min intervals during the first 10 min and subsequently (if necessary) at hourly intervals. When 500-1000 spores have been deposited, remove the spore-producing tissue, add a drop of sterile distilled water to the spore deposit and disperse the spores thoroughly with a sterile, bent glass rod. The basidiospores must be well dispersed on the medium so that the germlings will be well separated. Incubate the spread spores for 18-24 hours in the case of *S. commune*, or for 24-36 hours for *P. betulinus*. In either case, the plates should be incubated at 23^{o}-25^{o}C; incubation at temperatures in excess of 25^{o}C drastically reduces the viability of spores of *P. betulinus*; lower temperatures delay germination. The isolation of single spores may be undertaken when the majority of the germlings have reached a length of 30-50μ.

A fine sewing needle, ground to a chisel point and mounted in a pin vise or wooden handle, is a convenient instrument for cutting tiny blocks of agar that carry single germlings and for transferring these to suitable containers. The isolation of germlings can best be done under a dissecting microscope with *oblique* illumination. (Raper, 1963, described a mechanical device that greatly facilitates the isolation of germlings.)

The following table provides data for both species on the approximate duration, under laboratory conditions, of major stages of the respective life cycles.

Stage	*S. commune* 23°C	*P. betulinus* 25°C
Germination of basidiospores	18-24 hr	24-30 hr
Growth of germlings (to 1 cm)	3-4 days	4-6 days
Formation of heterokaryons	4-6 days	7-10 days
Fruiting of dikaryons	7-10 days	18-21 days

Mycelial Interactions: Homokaryon X Homokaryon

In *P. betulinus*, as in all bipolar forms, determining mating types among the progeny of a single fruiting body is simple. The simplest procedure--satisfactory if all the isolates interact normally--is the following. Select as tester a single monosporous isolate, the mating type of which is arbitrarily designated, e.g., A_1, and mate it with other isolates of the sample. Transfer a tiny bit of mycelium of the tester (1 mm^3 inoculum is ample) to the center of a plate of MEM medium, and place, 2-3 mm away, a corresponding bit of mycelium of an isolate to be tested. Repeat for each member of the sample. Incubate at 25°C for 7 days and examine the aerial hyphae 3-4 mm from the peripheries of the resulting colonies for clamp connections. The A_1 tester strain will have dikaryotized all A_2 isolates of the sample, and no discernible reaction will have occurred in matings with the A_1 isolates. Confirmation of the identity of A_1 isolates may be achieved by selecting an A_2 isolate and repeating the test with those isolates giving no reaction in the first series of matings. In actual practice, however, there is usually considerable variability among the isolates of a fruiting body collected from nature, and not all isolates can be expected to interact normally. It is, therefore, advisable to select a small sample of no fewer than six isolates and mate each with all others. The interactions,

	A_1	A_2
A_1	—	*dik*
A_2	*dik*	—,

will define two mutually exclusive mating types. From this series of matings, A_1 and A_2 testers can be chosen for scoring additional isolates.

In *S. commune* and other tetrapolar forms, the determination of mating types among the progeny of a single fruiting body is somewhat less direct. This follows from the fact that the common-*B* and common-*AB* interactions are often superficially similar and difficult to distinguish from each other. Unambiguous identification of all mating types thus involves either: (a) Mating the members of the sample in all possible paired combinations (a laborious business for all but tiny samples); or (b) two successive series of matings. Practicality dictates the choice between the two methods, here outlined for a sample of 10, the smallest sample that will be reasonably sure (P= .95) to provide at least one isolate of each mating type.

Method One For each of the 45 pairs of isolates, place small mycelial inocula 2-3 mm apart at the center of a plate of CYM medium. Incubate at 20°-25°C for 7 days, examine peripheral hyphae of the resulting colonies for evidence of heterokaryotization (see following section), and score each as "*dik*" (dikaryon), "A=" (common-*A* heterokaryon), or "—" (neither dikaryosis or common-*A* heterokaryosis). Arbitrarily assign a specific *A* and a specific *B*, e.g., A_1B_1, to a single isolate of the sample and identify the other isolates by means of the two recognizable interactions:

	A_1B_1	A_1B_2	A_2B_1	A_2B_2
A_1B_1	—	A=	—	*dik*
A_1B_2	A=	—	*dik*	—
A_2B_1	—	*dik*	—	A=
A_2B_2	*dik*	—	A=	—
A_xB_1	—	*dik*	—	*dik*

The *A* and *B* factors of *S. commune* are each constituted of two distinct loci, and these may recombine to yield nonparental factors of either series (the frequency for recombinant *A* factors may be as high as 20%). Since recombinant factors are compatible with both parental factors of the same series, the result illustrated (for the *A* factor) in the last line of the scheme above is not unexpected even in modest samples.

Method Two It is evident from the scheme above that mating all members of the sample with a single tester will identify isolates of two of the four basic mating types. The results of such a preliminary test will provide the necessary tester strains for the positive identification of the remaining isolates:

Preliminary test			Testers	
Isolates		A_1B_1		
	A_1B_1	—		
	A_1B_2	A= - - - - - - - - - - - -		↓
	A_2B_1	—		
	A_2B_2	*dik* - - - - - →	A_2B_2	A_1B_2
Definitive test				
	A_1B_1		*dik*	A=
	A_2B_1		A=	*dik*

This labor-saving procedure, however, is not sufficiently discriminating to identify strains carrying recombinant *A* or *B* factors.

If recombinant factors are to be recognized in a somewhat larger sample, Method One is recommended for a small sample to establish the four basic mating types, all four of which should then be used as testers against each of the remaining isolates. Recombinant *A*'s and *B*'s will be revealed by these tests, but if specific identification of each recombinant is of interest, additional tests are required, here illustrated for the two reciprocal *A* recombinants:

	A_xB_1	A_yB_1
A_xB_2	A=	*dik*
A_yB_2	*dik*	A=

The "new" factors originating here via intrafactor recombination are identical to factors that occur in other stocks of *S. commune* in nature. There are extensive series of *A* and *B* factors in the natural population, and all factors of either series are alternate and equivalent. Thus, while samples of progeny collected from two fruiting bodies collected at different places will *each* contain four mating types, the probability that there will be no common mating type in the two samples is about 98%.

Characteristics of Heterokaryons

The two heterokaryons of *P. betulinus* (dipolar) and the four heterokaryons of *S. commune* (tetrapolar) are very characteristic as regards several features: (a) Mycelial morphology and vigor, (b) hyphal morphology, (c) heterokaryotic stability, (d) detailed nuclear distribution, and (e) the type of septation. The following descriptions are limited to the more evident, distinguishing features that will be of greatest utility in the recognition of the various interactions among homokaryons. The more detailed features of nuclear distribution and septation of the homokaryon and three types of heterokaryons of *S. commune* are diagrammed in the accompanying figure (Figure 27).

Compatible These heterokaryons have unlike factors of one series ($A_1 \times A_2$, bipolar) and unlike factors of both series ($A_1B_1 \times A_2B_2$, tetrapolar). Matings that are heteroallelic for both incompatibility factors react to establish the dikaryon, a specialized type of heterokaryon in which the nuclei of the two parental strains become associated in pairs and divide synchronously by means of conjugate division; the ratio of the nuclei of the two parental strains is accordingly maintained at an exact 1:1. Clamp connections, small buckle-like structures, are formed at all septa in association with conjugate division and are characteristic of the dikaryon. The dikaryon is capable of indefinite vegetative growth and eventually produces the fruiting body upon which are borne the basidia, within which nuclear fusion and meiosis occur to complete the sexual cycle.

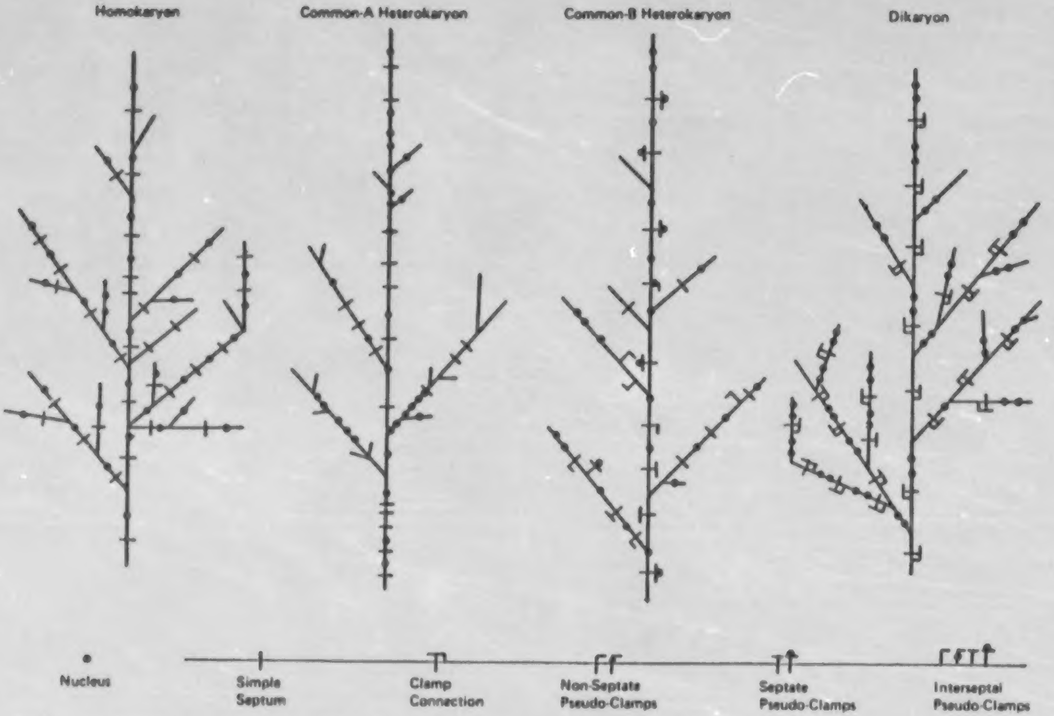
Homokaryon
Common-A Heterokaryon
Common-B Heterokaryon
Dikaryon
Nucleus
Simple Septum
Clamp Connection
Non-Septate Pseudo-Clamps
Septate Pseudo-Clamps
Interseptal Pseudo-Clamps

FIGURE 27 *Nuclear distribution and septation in S. commune.*

Hemicompatible A These heterokaryons have like factors of the *A* series and unlike factors of the *B* series (A_1B_1 x A_1B_2, tetrapolar). Homokaryons carrying common *A* factors interact to yield a bizarre type of heterokaryon originally termed "flat." Macroscopically it is a sparse, depressed growth as compared to either the homokaryon or the dikaryon, and microscopically it is distinguished by gnarled, irregularly branched hyphae and frequent extrusion of protoplasm from the cells. Heterokaryosis, here, as in the dikaryon, extends throughout the pre-existing mycelia of both mates and is propagated by continuing vegetative growth.

Hemicompatible B These heterokaryons have unlike factors of the *A* series, like factors of the *B* series (A_1B_1 x A_2B_1, tetrapolar). Interaction between homokaryons carrying identical *B* factors results in the establishment of a limited heterokaryon in the immediate vicinity of the line of intermingling of the two mates. Locally, this heterokaryon resembles the dikaryon, but has pseudo-clamps at the septa (pseudo-clamps are incompleted clamps by failure of fusion of the hook cell to the subterminal cell). The common-*B* heterokaryon differs from the dikaryon in that the heterokaryon does not extend throughout the pre-established mycelia of the mates and in its relatively greater instability.

Noncompatible These heterokaryons have like factor of one series (A_1 x A_1, bipolar) and like factors of both series (A_1B_1 x A_1B_1, tetrapolar). Normal homokaryons of the same mating type give no discernible interaction when mated. Certain special circumstances, however, such as unlike biochemical deficiencies carried by the two strains, will allow an interaction to occur that permits growth in this case upon a minimal medium. The heterokaryon thus formed, however, closely resembles the homokaryon and is distinguishable from a homokaryon only by special tests.

The interactions leading to common-*B* and common-*AB* heterokaryons may not be readily distinguishable, unless a characteristic line of sparse growth (the barrage) forms between the two mates of the common-*B* mating. In any event, the heterokaryotic products of both crosses will not be recognizable in matings of normal strains on complete media. These heterokaryons, however, may be readily isolated from matings between strains carrying nonallelic auxotrophic mutations. From matings of the appropriate strains, remove series of small blocks of mycelium-agar from the lines of confrontation between the mates and transfer to minimal medium. The resulting mycelia will be the nutritionally forced heterokaryons.

Mycelial Interactions: Dikaryon x Homokaryon

Reactions comparable to those above between homokaryons also occur between heterokaryons and homokaryons. Hyphal fusions appear to occur as a matter of course between any two mycelia of the same species; following fusion between a heterokaryon and a homokaryon, subsequent developments are determined by the interplay of incompatibility factors in the two mycelia.

The interaction between dikaryons and homokaryons was first described by Buller in the 1930's and has since become known as the Buller Phenomenon. There are three possible interrelations here that may be classified as follows:

Legitimate

Compatible: Both components of dikaryon compatible with homokaryon, e.g., $(A_1B_1 + A_2B_2) \times A_3B_3$.

Hemicompatible: Only one dikaryotic component compatible with homokaryon, e.g., $(A_1B_1 + A_2B_2) \times A_1B_1$

Illegitimate

Noncompatible: Neither dikaryotic component compatible with homokaryon, e.g., $(A_1B_1 + A_2B_2) \times A_1B_2$

Dikaryosis of the homokaryon occurs readily and regularly in compatible and hemicompatible combinations; dikaryosis also occurs in most noncompatible combinations, but usually after some delay and only in isolated portions of the homokaryotic mycelium. This has been shown to occur as the result of somatic recombination that yields nuclei of new, compatible mating type:

$$\begin{array}{c} (A_1B_1 + A_2B_2) \quad \times \quad A_1B_2 \\ \downarrow \\ (A_1B_2) + A_2B_1 \longrightarrow A_2B_1 + A_1B_2 \end{array}$$

The occurrence of somatic recombination in noncompatible matings means, of course, that *all* dikaryon-homokaryon combinations are sexually fertile.

Two of the three types of dikaryon-homokaryon matings listed above can be easily established among the strains identified among the progeny of a single fruiting body. To accomplish this, make a mating of strains of A_1B_1 x A_2B_2 to establish a dikaryon. The dikaryon is then mated with a strain of each of the four mating types, (1) A_1B_1, (2) A_1B_2, (3) A_2B_1, (4) A_2B_2. In each case, place the inoculum of the dikaryon 2 cm from the edge of a plate of CYM and the inoculum of the homokaryon on the same radius and 1 cm nearer the center of the plate. Examine the plates at five days and thereafter; observe the reactions that occur in the homokaryotic mates and compare these reactions with those observed in matings between homokaryotic strains. Matings (1) and (4) are hemicompatible (COM), while matings (2) and (3) are noncompatible (NON).

Small sectors of dense dikaryotic mycelium may appear in the homokaryotic matings of (2) and (3) after about 10 days. These may originate either (a) by the migration of *both* nuclei of the dikaryon through the homokaryon and the reestablishment of the original dikaryon or (b) by the dikaryotization of the homokaryon by a compatible nucleus resulting from somatic recombination. A distinction can be made between these two processes by the determination of the genotype of the derived dikaryon in matings with homokaryotic testers (illustrated below for mating with A_1B_2):

Dikaryon-Homokaryon mating $(A_1B_1 + A_2B_2)$ x A_1B_2 ↓	Mating Types			
	A_1B_1	A_1B_2	A_2B_1	A_2B_2
(a) Re-establishment of original dikaryon: $(A_1B_1 + A_2B_2)$	COM	NON	NON	COM
or ↓				
(b) Dikaryotization of homokaryon by recombinant nucleus: $(A_2B_1 + A_1B_2)$	NON	COM	COM	NON

Induction of Mutations

Genetic work with Homobasidiomycetes beyond the basic clarification of patterns of sexuality, mycelial interactions, and characteristics of heterokaryons depends, as with other types of organisms, upon the availability of genetic markers. Few usable markers, other than the incompatibility factors themselves, have been found in strains isolated from nature, and it is accordingly necessary to generate them in the laboratory.

These forms present one difficulty that happily is shared by only very few fungi: Most species of this group lack asexual spores that in other forms provide large, genetically homogenous populations of cells for mutagenic treatment. It has accordingly been necessary to develop procedures that utilize mycelial macerates instead.

The strain to be treated is inoculated into liquid medium (1-2 cm deep) and allowed to grow, without shaking, to provide a sufficient quantity of vigorously growing mycelium. The culture fluid is then decanted and the mycelium transferred to the cup of a Waring blender containing the minimal quantity of liquid to cover the blades (semi-micro cup recommended). Maceration for 1-2 min at high speed is usually sufficient to reduce the mycelium to a population of fragments of varying size and nuclear content (overheating of the blender cup must be avoided). The macerate may be washed by centrifugation, resuspended in any desired liquid, and treated as any other suspended population. With *Schizophyllum commune* and *Polyporus palustris*, a bipolar form closely related to *P. betulinus*, such macerates usually contain from 200-2000 viable fragments per ml per unit optical density at 660 mμ. *P. betulinus* does not yield satisfactory macerates.

Of the numerous mutagenic agents available, N-methyl-N'-nitro-N-nitrosoguanidine or NG (available from the Aldrich Chemical Co., Milwaukee, Wisconsin, catalog #M6200) has proven efficient in the induction of auxotrophic and morphological mutations in *S. commune* and in *P. palustris*. Two different protocols for the two species follow, but either protocol could be used with any material with the necessary adjustment of dosage, different organisms differing widely in their sensitivity to NG.

Short Exposure with S. commune

- Preparation of macerate--Grow mycelia in a complete liquid medium. Macerate. Centrifuge and resuspend macerate in 0.2 M citrate buffer, pH 5.0.
- Preparation of NG solution--Dissolve mutagen in 0.2 M citrate buffer, pH 5.0, at a concentration of 4.0 mg/ml. (This concentration approaches the limit of solubility of NG in this solvent and some heating is needed to effect solution.) NG is labile and cannot be autoclaved or stored for extended periods. The solution is ordinarily auto-sterilizing, but certain sterilization may be achieved by passing the solution through a millipore filter. Prepare minimal quantities and use them as quickly as practical.
- Exposure of macerate--Add 0.25 ml of NG solution/ml of macerate and allow to stand without shaking during treatment period. Plate to complete medium to terminate treatment. If plating is done at high dilution, no further manipulations are needed. If plating is done at high density, the reaction mixture should be centrifuged and resuspended in neutral buffer before plating.

With *S. commune*, this protocol will yield an exponential killing curve with a half-killing time of about 30 min.

Long Exposure with P. palustris

- Preparation of macerate--Grow mycelia in liquid complete medium. Wash mycelia in 0.2 M citrate buffer, pH 5.0. Resuspend in same buffer, and macerate.
- Preparation of NG solution; treat as above.
- Exposure of macerate--Add aliquots of NG and macerate to molten (46°C) complete agar and pour plates immediately. The NG will hydrolyze and induce mutations during incubation. A final NG concentration of 2.5 μg/ml constitutes an L.D. 50 for *P. palustris*.

Recovery and Identification of Mutations

Either of the above treatments should yield 5-10% morphological mutations and 2-5% auxotrophic mutations at about 50% killing. If plating density is appropriate (about 100 surviving fragments per plate), morphological mutations will be recognizable on the dilution plates and may be directly isolated. Auxotrophic mutations cannot be so easily recognized, and, unfortunately, no successful technique is known for their enrichment; their recovery and recognition are accordingly somewhat laborious. Tiny bits of mycelia (single hyphal tips would be ideal) are taken from individual colonies and transferred to plates of complete medium, where they are allowed to grow for a few days. Each is then simultaneously subcultured with the smallest practical inocula to complete and minimal media, where failure of growth on minimal medium reflects auxotrophy. Since NG is an extremely potent mutagen, any desirable mutation, morphological or auxotrophic, will probably be associated with additional and undesirable genetic ills. It is therefore generally necessary to cross the original mutant strain with a wild mate, from the progeny of which cross strains that are wild-type for all but the desired trait can be selected. This cross also provides an opportunity to obtain the mutant trait in strains of different mating types.

The identification of auxotrophic mutations may be made either prior to or subsequent to the "clean-up" cross. A great majority of all auxotrophic mutations are identifiable with relatively few specific substances, and screening with the following 20 compounds should account for more than 90% of recovered mutants unable to grow on minimal medium: biotin, choline, inositol, niacin, *P*-aminobenzoic acid, Ca-pantothenate, pyridoxin, riboflavin, arginine, histidine, leucine, lysine, methionine, cysteine, phenylalanine, proline, serine, tryptophan, adenosine *or* guanosine, and uridine *or* cytidine. These substances should be used in the following final concentrations; amino acids, 10 μg/ml; purines and pyrimidines, 1 μg/ml; and vitamins, 0.1 μg/ml.

Numerous schemes for the efficient screening of auxotrophic mutations have been reported; the following simple procedure is probably as good as any. The mutant strain(s) is inoculated to a series of five plates of minimal medium, each supplemented with four of the substances to be tested. With rare exception, each mutant will grow only upon a single medium, and specific identification of the mutation can then be made by similarly testing the four individual components of the initial group.

Mutations may affect the synthetic competence for a specific compound to varying degrees, and, as a consequence, auxotrophic mutations will range from total inability to subnormal ability to grow on minimal medium. The latter, "leaky" mutations, are generally less desirable than absolute mutations for many purposes, e.g., nutritional forcing of heterokaryosis, etc., but they can be very useful as genetic markers if they can be readily recognized.

See Raper and San Antonio, 1954; Raper and Miles, 1958; Raper *et al.*, 1958; Snider and Raper, 1958; Raper, 1966.

METHODS FOR *COPRINUS* GENETICS

The Hymenomycetes amenable to laboratory studies have two features of special interest to the geneticist: The dikaryon organization and, in the heterothallic forms, the outbreeding system that controls the dikaryon's establishment. A third feature, almost totally neglected by geneticists, is the complex fruit body that provides opportunity for the study of the genetic control of morphogenesis.

The advantages of *Coprinus lagopus* are that the basidiospores are large (10x6μ) and black and therefore relatively easy to isolate either at random or in tetrads; that the homokaryon produces uninucleate oidia that may be used in mutant hunts or for isolating diploid strains; and that markers on six of the ten chromosomes have been identified and mapped.

In other respects such as ease of culture, speed of fruiting or demonstration of the chief features of tetrapolar sexuality, *Coprinus* may be more or less convenient than for example *Schizophyllum commune*.

The species described here is called *Coprinus lagopus* after Buller's (1924) account of it. The taxonomically correct name is probably *C. cinereus* (Orton, 1957) although *C. fimetarius* has been widely used. *C. lagopus* (sensu Buller) can generally be found in Europe on manure heaps particularly during the cool wet months. Buller recovered the fungus from horse dung balls, collected in Winnipeg, which were kept for a few weeks in a large glass chamber in the laboratory. Several workers in England and the United States keep stocks of wild type and mutant lines. To ensure a common genetic background, most mutants have been isolated from the wild type homokaryon H9 isolated in Hertfordshire, England, in 1957.

Culture and Storage of Materials

Slant cultures of homokaryons and dikaryons remain viable at 40°C for periods of a year or more. To prevent drying out, screw-cap vials with moisture-tight liners may be used. The cap should be slackened off 1/4 or 1/2 turn before incubation. If cotton plugs are used, culture tubes may be sealed with "parafilm." Long-term storage of slants under mineral oil is also useful.

Basidiospores from freshly autolyzed fruit bodies can be stored in small screw-cap vials of silica-gel sterilized in a hot air oven. One-hundred mesh silicic acid (chromatography grade) is best and the vials need contain no more than 1-2 ml of the powder. Pieces of gill thoroughly mixed in the silica-gel with a sterile rod make a satisfactory basidiospore preparation for storage. Oidia suspended in water in a concentration of 1×10^6/ml or greater are mixed with an equivalent volume of sterile nonfat milk. Approximately 0.25 ml of such a suspension is added to the silica-gel. The tubes are stored at 4°C.

Complete medium (M-94b) is used for routine culturing of wild type and mutant strains and for mating type determination. A variety of natural media including potato glucose agar and malt extract agar support good mycelial growth. A minimal medium (M-94a) devised by Fries (1953) supports vigorous growth of wild type at temperatures up to 40°C. Minimal and complete media are usually made up in 2- 4-liter batches. The pH of both media after autoclaving is 6.8. The media are solidified with 2% agar. To avoid caramelization and precipitation during preparation of liquid minimal medium, the trace elements and sugar should be autoclaved separately and added aseptically to the other ingredients when cool. For this it is convenient to use a 12.5% glucose solution and the undiluted trace element solution and to make up the other ingredients using the amounts for 1 liter in 800 ml. These are combined in the proportions 4:1:20, respectively.

Minimal medium may be supplemented with the individual requirements of auxotrophic mutants by the addition of: Amino acids, 100 mg; purines and pyrimidines, 10 mg; and vitamins in the following concentrations per liter--riboflavin 50 mg, *P*-aminobenzoic acid 50 mg, nicotinic acid 200 mg, calcium pantothenate 200 mg, pyridoxin 50 mg, choline chloride 200 mg, inositol 400 mg, biotin 0.1 mg, distilled water to 1 liter. The mixture, or individual vitamins in above concentration, are used at the rate of 10 ml per liter of minimal medium.

Substitution of equimolar nitrogen in the form of KNO_3 for asparagine and ammonium tartrate in liquid minimal medium reduces mycelial dry weight by a factor of 7.5 at 37°C.

Cultures are incubated at 37°C, although exceptions are noted elsewhere for fruiting cultures and for mating type tests. Maximal yields of mycelium in liquid culture are obtained when the medium is shallow and not agitated. A convenient depth is obtained by using Erlenmeyer flasks containing not more than 1/10 their capacity of medium. Shaken or aerated cultures give unsatisfactory, pelleted growth. The most convenient inoculum is a blended agar culture fine enough to be pipetted.

Basidiospore germination on minimal and complete medium is improved by the addition of .01% furfural (Emerson, 1954). The furfural is most conveniently added prior to melting for pouring plates and does not need to be sterilized. Heat shock treatment (30 min at 60°C) as used to induce ascospore germination in *Neurospora crassa* is lethal for *Coprinus* basidiospores. Dung decoction agar (M-11) has also been used to improve spore germination.

Basidiospore suspensions are made by shaking fresh or dry gill fragments, or spores stored in silica-gel, in small screw-cap vials of sterile distilled water.

Spore density is estimated with a haemocytometer. Random germinated basidiospores can be isolated by spreading approximately 2,000 basidiospores per plate in 0.2 ml of suspension. At this density single germinated spores should be removed on small blocks of agar with a fine needle under a dissecting microscope after 18-24 hours' incubation. To obtain macroscopic colonies, fewer (50-100) basidiospores should be spread and incubated for up to 48 hours. If furfural is used in the medium, growth is stunted and abnormal.

Oidia are produced in abundance by most mycelia except the dikaryon and common-*B* heterokaryon. Since oidia are uninucleate, they are useful for isolating mutants from haploid homokaryons and for recovering diploids from common-*A* heterokaryons.

A suspension of oidia is made by flooding the surface of a 3-10 day-old mycelium with sterile distilled water and gently rubbing it with a glass spreader. The suspension should be filtered to remove hyphal fragments either by withdrawal through a sterile pipette the tip of which touches a small piece of sterile absorbent cotton placed in the suspension, or by decanting into a small sterile funnel containing a thin pad of glass wool. Densities of oidial suspensions are estimated with a haemocytometer. The germination of oidia is erratic; frequently less than 20% give rise to colonies.

Mutants

Auxotrophs The most widely used mutagen has been UV. Oidial suspensions of density about 1×10^6/ml in water are usually treated for various times resulting in survival rates from 5% to .01%. Of the various enrichment methods tried, filtration enrichment (Day and Anderson, 1961) appears to be best, although mutant yields are frequently far from satisfactory.

Morphological and Other Mutants Mutants with altered colony morphology (dwarf, sparse, no aerial mycelium, etc.) are often encountered in mutation experiments. Little attention has been paid to these forms because of the difficulty of recognizing multiple mutant phenotypes and because they do not possess the selective advantages offered by auxotrophs. Some, however, are of considerable intrinsic interest. For example, the oidial mutant produces extremely large numbers of oidia even on submerged hyphae and behaves as a recessive with wild type in common-*A* heterokaryons. Dikaryons homozygous for this character bear no oidia and do not fruit.

Some haploid homokaryons with mutant *A* factors will form fruit bodies when inoculated onto sterile dung (Day, 1963). The study of fruit body morphogenesis by genetic blocks might well be attempted in such stocks.

A stock list of more than 80 different original mutants and other wild type and recombinant lines has been prepared by David Moore, as well as a linkage map (Moore, 1967).

Meiotic Analysis

C. lagopus is tetrapolar, and fertile dikaryons are normally produced only by mating two stocks carrying different *A* and *B* factors (e.g., A_1B_1 x A_2B_2). Inocula of the two compatible strains are placed either to touch or within a few millimeters of each other on the surface of complete medium in a Petri dish. The dish bottom should be marked so that the two parents can be identified. Growing hyphae from each parent anastomose, and dikaryotization occurs by nuclear migration. The dikaryotic growth that appears within 24-48 hours is readily distinguished by its clamp connections, acute angled branching habit, and rapid growth rate that is approximately twice that of a homokaryon (Lewis, 1961).

Normally, both parents are dikaryotized as a result of nuclear migration and both can serve as the source of dikaryon inoculum. It should be noted, however, that the two dikaryons are reciprocally constituted, having identical nuclei but not necessarily identical cytoplasms (Day, 1959). Some haploid homokaryons, called unilateral maters, only donate nuclei and do not become dikaryotized themselves.

Flasks of fruiting medium (M-94c, d) inoculated with a dikaryon, incubated in the dark for two days at 37°C and then placed either near a laboratory window at room temperature or in an incubator at 26°-27°C in light will produce fruit bodies in 7-10 days. Fruiting will not take place at temperatures above 28°C nor in continuous darkness. In natural daylight conditions, fruit bodies are frequently fully expanded and begin discharging spores in the evening. Soon after maturation the cap undergoes rapid autolysis. Tetrad isolation is feasible only during the period of spore discharge, which lasts three or four hours. While removal to a refrigerator (about 4°C) will prevent autolysis for periods up to 8 hours, fruit bodies stored in the cold for more than two to three hours rarely resume normal spore discharge and are thus unsuitable for direct tetrad isolation by micromanipulator. A convenient way of ensuring a supply of ripe fruit bodies near a given time of day is to adjust a 14-hour light period so that it ends some 3-4 hours before that time. The timing of the light period should be the same throughout the post 37°C incubation period of the inoculated dung.

Tetrad Isolation Direct isolation requires a micromanipulator and also the preparation of fruit body material at the right stage of development. As a fruit body matures, the cap expands and the gills separate prior to spore liberation and autolysis. The fruit body should be picked before autolysis has begun and the cap cut in half longitudinally. Parts of single or small groups of gills may be lifted off with fine forceps applied through the cap tissue--the free gill edge is too fragile to be handled in this way. These pieces are then laid flat with the hymenial surface uppermost, on one side of an agar rectangle mounted on a microscope slide. The hymenial surface has a black silken sheen easily distinguished from the inner surface of a split gill.

The slide, placed in a dish lined with moist filter paper, may be kept at room temperature until spore discharge (verified by observation under low power) begins. Basidiospores in tetrads, about to be discharged, will adhere to a glass micro-needle. Because of the high density of tetrads ($3000/mm^2$), the micro-needle must be held in a micromanipulator. Suitably isolated tetrads are often found on or near the gill edge. The tetrads are put down in rows on the free agar surface, alongside the gill, using the mechanical stage of the microscope. The individual spores of each tetrad are then spread apart sufficiently so that they can be cut out on small blocks of agar, with a fine hand-held needle, under a steroscopic microscope.

A particularly useful needle for this type of transfer is made by sharpening tungsten wire (30 gauge) in molten sodium nitrate heated in a porcelain crucible lid supported on a pipe-clay triangle. The tip of the wire held against the molten material is rapidly eroded. Tungsten needles can be repeatedly flamed. They are mounted either in the end of a length of 1/8" aluminum rod in a saw cut that is then closed with a vise, or in a ready-made needle holder.

Moore (1966) has used a simpler method of tetrad isolation in which the gill is placed directly on a glass slide and allowed to dry. If the surface is then swept with a micromanipulator needle, among the spores on the glass there is a substantial number of tetrads. The four spores of a tetrad frequently clump together, forming a unit when the tetrad collapses during drying. The tetrads can be isolated and the basidiospores allowed to germinate when convenient. Individual basidiospores from random isolations or tetrads may be transferred to plates of complete medium, 16 per dish. These should not be incubated more than 48 hours before transfer to test plates, or confluent growth will result. The dishes may be stored at 4°C for 3-4 weeks.

Unfortunately, there is no satisfactory method of replica plating for *Coprinus*.

Mating Type Determination Haploid homokaryotic colonies from basidiospores from the same fruit body are generally of four mating types. These can be distinguished by test matings with two tester stocks as shown below:

	Progeny of A_1B_1 A_2B_2			
Tester	A_1B_1	A_1B_2	A_2B_1	A_2B_2
A_1B_3	-	-	+	+
A_3B_1	-	+	-	+

+ = dikaryon formation; - = no dikaryon

The choice of which allele, A_1 or A_2, B_1 or B_2, each tester carries is arbitrary. In *Coprinus*, unlike *Schizophyllum commune*, incompatibility due to common-*A* is not readily distinguishable from incompatibility due to common-*B*. If the progeny are to be screened for nonparental or recombinant factors, then both parental alleles must be employed. Thus tester stocks A_2B_3 and A_3B_2 are used as well. If testing can be done in two stages so that only stocks compatible with, say, A_1B_3 are tested with A_2B_3, some labor can be avoided.

Since some unknowns may be unilateral maters, care should be taken to ensure that the tester stocks selected are not unilateral but will produce vigorous dikaryons that can be recognized even without the use of a microscope.

Tester stocks are best grown in plate culture and when used for testing cut into small blocks of 1-2 mm^2. They may be stored at 4°C for up to 5-6 weeks. The blocks of the tester are spaced 16/9-cm dish of complete medium and inocula of the unknowns placed alongside to touch. After 36 hours' incubation at 37°C, or 48 hours at 27°C, matings can be scored. Until some experience has been gained, or if the test is crucial, it is advisable to examine each mating under low power to look for clamps on fringe hyphae lying on the surface of the agar on the tester side. No cover slip is needed. Clamps are much more difficult to see on aerial and submerged hyphae.

Heterokaryons and Diploids

Common-A Heterokaryon This heterokaryon has no distinctive morphology and produces abundant oidia like the homokaryon (Swiezynski and Day, 1960). It is best prepared by mating two different complementary auxotrophic strains on minimal medium. The resultant prototrophic growth can be tested for heterokaryosis in two ways:

- It should be compatible with both tester stocks carrying the parental *B* alleles.
- Both markers should be recovered among single oidia but only very low frequencies (10-5 or less) of prototrophs.

Common-B Heterokaryon Like the Common-*A* heterokaryon, this is best prepared on minimal medium from complementary auxotrophic strains, since it is otherwise very unstable. The hyphae have false clamps at many cross-walls. No detailed cytological investigation has been made of this mycelium, but it appears that only the tip cells are heterokaryotic, the remaining mycelium being largely a mixture of the component homokaryons.

De-dikaryotization The homokaryotic components of dikaryotic mycelium of *Coprinus* may be recovered by a method first described by Lewis (1961). Mycelia of *Coprinus* produce swollen thick walled cells called chlamydospores. These are borne just below and at the surface of solid media especially in older mycelia. They can be conveniently prepared in suspension in a ground glass homogenizer and a high proportion germinate when plated onto fresh medium. Chlamydospores from a dikaryon are usually dikaryotic and germinate at one end. They sometimes germinate at both ends and may produce mycelia that are homokaryotic and without clamps. Some homokaryotic chlamydospores are formed by dikaryons and these are also recognized by their unclamped outgrowths. The homokaryotic hyphae must be transferred before they contact others or they become dikaryotized. Cowan (1964) has shown that homokaryons may be recovered from the veil cells of fruit bodies. The veil cells are macerated and broken apart in a glass homogenizer.

Diploids Stable diploid strains may be produced by the method described by Casselton (1965), or by selecting unreduced basidiospores (Day and Roberts, 1969). Oidia are harvested from a common-*A* heterokaryon prepared from doubly-marked stocks. The markers must be complementary so that the heterokaryon is prototrophic. The oidia are plated on minimal medium at densities of from 2-4x10^6 per plate. After incubation for 3 days, prototrophic colonies appear and may be isolated. These are tested for diploidy by plating a small sample of the oidia they produce on minimal medium. Diploid oidia will give rise to as many prototrophic colonies as viable oidia sown, while a heterokaryon will not. The diploid prototrophs will also mate with both *B* testers.

24 Special Materials

ASCUS STRUCTURE

Ascus morphology has become an increasingly important character in pyrenomycete taxonomy in recent years, especially in the delimitation of higher categories. The nature of the ascus wall, i.e., whether it is bitunicate or unitunicate, forms one criterion for the separation of the pyrenomycetous Ascomycetes into subclasses. In general, though, ascus structure has been used only incidentally in the separation of lower taxa. Recent studies, however, have included observations on the structure of the apical apparatus of the ascus as well as on other morphological characteristics of the ascocarp. Chadefaud and his students in France have emphasized this aspect of pyrenomycete morphology, and from this they have evolved a taxonomic scheme that divides the Pyrenomycetes on this character rather than on the nature of the ascus wall.

Studies on ascus structure can be conducted on either fresh or dried material. To obtain good results it is essential to remove all of the ascocarp wall material from the mount so that well-flattened preparations can be obtained. Material should be observed in water first to determine what structures are visible in unstained preparations; e.g., the "refractive rings" typical of members of the Diaporthales. Stains can easily be added by placing a drop of the stain solution alongside the cover slip and allowing it to diffuse into the material. Progressive color changes can be observed this way, although the time required for staining varies with the material and the stain being used. Generally, though, staining occurs fairly quickly. A wide variety of dyes has been utilized in ascus studies, but the best results have been obtained with a few standard ones. Chadefaud uses cotton blue in lactophenol to study the structure of bitunicate species. The inner wall layer (endotunica) stains, whereas the outer wall (ectotunica) is quite resistant to stains. The endotunica is also stained by other dyes, such as phloxine and ink. Mention should perhaps be made here of a report of a multilayered endotunica in *Botryosphaeria* and *Pleospora*. This layering is visible in mounts made in potassium hydroxide, and shows up best in asci that have been ruptured by pressure on the cover slip.

The solutions used most commonly in studying unitunicate asci are iodine ($KI+I_2$), cotton blue, and blue-black ink. Janus green, Congo red, and ruthenium red have also been used, but with less satisfactory results than are obtained with the blue dyes. Asci that have structures that stain in iodine are said to be amyloid positive. Cotton blue and the blue-black inks give the best results with most material, although not all species stain equally well with both dyes. Waterman's blue-black ink is usually specified, but Carter's midnight-blue is also said to be good. Both cotton blue and ink give a range of stain intensity from light blue to deep blue in different areas of such complex ascus tips as are found in the Xylariaceae. The age of the asci observed is important, since differentiation of the ascus tip occurs as the ascus matures.

In general, the red dyes stain less intensely than the blue ones, and they also have less contrast and thus are not as well suited for making observations. The violet dyes do not work well. Occasionally a particular stain will work well for a given species: the doughnut-shaped ring present in asci of *Nectria gliocladioides* shows up well in nigrosin, but nigrosin usually does not work well for other species.

All of the dyes should be those certified for biological or histological use and made up as 0.5% or 1%

aqueous solutions. With most dyes, the lower concentration is adequate, and for some even more dilute solutions are desirable. Distilled water is used and solutions are filtered into dropping bottles for use. Although most of the commonly used stains can be stored for several months, some, such as nigrosin, break down and must be made up fresh.

The Prototunicate Ascus

The prototunicate ascus is thin-walled and dissolves early, releasing ascospores within the ascocarp. It does not have a differentiated apparatus for forcible discharge of ascospores. Young cultures of *Chaetomium* would be suitable for the demonstration of this type of ascus. Overmature material may not have asci present and the unwary may mistake such an ascocarp for a pycnidium bearing conidia.

The Bitunicate Ascus

The bitunicate ascus consists of an outer wall, called the ectotunica, and an inner wall, the endotunica. The ectotunica is thin, about 0.5μ, rigid, nonhydrophylic, and difficult to stain. The endotunica is thicker, extensible, hydrophylic and readily stained. For ascospore discharge the ectotunica breaks near the apex and the endotunica elongates and discharges the ascospores. Chadefaud uses the terms "nassasce" and the highly appropriate "Jack-in-the-box" for the bitunicate ascus. The term nassasce is derived from the "nasse apicale," a group of pendant strands in the upper part of the ascus; it can be demonstrated by staining with 1% aqueous cotton blue but will often be beyond the capabilities of beginning students.

The bitunicate or Jack-in-the-box ascus can be demonstrated effectively using cultures of *Leptosphaerulina* spp., fresh collections of *Mycosphaerella* or *Pleospora*, and even dried specimens. Crush an ascocarp in water with needles until a colorless sphere comes out of the dark wall. Remove the wall debris and place a thin (#1) cover slip over the group of asci. Observe under low power objective and press slide until asci fan out like the spokes of a wheel. Blot away excess water and watch for rapid elongation of asci as the cover slip is gradually drawn down on the specimen during drying. When an ascus has elongated, change to high power and look for the broken ectotunica.

The bitunicate ascus can be demonstrated by staining with ammoniacal Congo red using the method from Richardson and Morgan Jones (1964), but this is less satisfactory than seeing the actual extension of the endotunica and fractured ectotunica as outlined above. With very old dried material the staining method may be the only suitable procedure.

A word of caution on the term bitunicate--its derivation indicates that the ascus has two walls. The definition, however, extends the meaning to cover the function in spore discharge (Jack-in the-box). Students will find ascomycetes with 2-layered asci that have an entirely different method of spore discharge. Van Brummelen (1967) describes and illustrates an ascus of *Ascobolus immersus* that has two layers, but these are fused together and the inner one does not extend. The bitunicate ascus has two *separable* walls, the inner one extending in spore discharge. The inner wall of the bitunicate ascus has been shown in at least a few species to consist of many layers (Funk and Schoemaker, 1967), but this substructure does not preclude the use of the term bitunicate.

The Unitunicate Ascus

Typically, a unitunicate ascus has one wall and a ring or annulus in the slightly thickened apical region. The annulus is involved in forcible discharge of ascospores. There are a number of variations in the apical structure of the unitunicate ascus.

The Amyloid Annulus The annulus is amyloid if it gives a blue color when mounted in a 1% solution of iodine in potassium iodide, I-KI, or in Melzer's Reagent. Examples of this can be found in many species of *Hypoxylon*.

The procedure is to section perithecia, tease out a group of asci in a drop of water on a slide, observe through a thin (#1) cover slip, and then add a drop of iodine solution at the edge of the preparation. The blue ring should be evident within a few seconds. Alkaline solutions like KOH will inhibit this reaction. Semipermanent slides can be made by adding lactic acid to the mount and sealing it with two layers of clear nail lacquer.

In members of the family Xylariaceae this plug or ring typically turns blue when stained with Melzer's reagent (I_2-KI). Although this character may be demonstrated adequately with dried material, live cultures may also be used. *Hypocopra merdaria*, *Poronia punctata*, and *Podosordaria leporina* are coprophilous members of the Xylariaceae that will fruit in the laboratory on a sterile dung plate. *Hypocopra* in particular shows a massive amyloid apical plug or ring. *Hypocopra merdaria* is frequently found on cow dung collected in the field; cultures may be obtained by surface-sterilizing perithecia with 100% Chlorox and placing them on a suitable nutrient agar. Within a week or so the mycelium should grow out from the perithecia and subcultures can be made. In order to obtain fruiting, inoculate on V-8 oat agar (M-3) at three separate places on each plate and incubate at room temperature for 2 weeks. When the mycelial mat has covered each plate, spread over the top a sterile cow dung paste enriched with the following vitamin solution: Biotin, 5 μg/liter; folic acid (suspended), 500 μg/liter; thiamin, 100 μg/liter. Note that effective sterilization of dung involves autoclaving for periods of up to an hour on each of three successive days. Within 2 weeks the mycelium should overgrow the dung; 5-6 weeks after application of the dung paste, mature perithecia will appear as separate, totally immersed bodies in the dung.

Fertile stromata of *Poronia punctata* and *Podosordaria leporina* can be obtained by following the same procedure and substituting horse dung or rabbit dung, respectively, for the cow dung. Here the time required for fruiting is less--3-4 weeks after application of the dung. Cultures can be maintained for long periods of time without loss of viability if the practice of using mature perithecia or fertile stromata as an inoculum for subcultures is adopted.

In organisms such as *Podospora* and *Neocosmospora*, solid mats of ascocarps form on the surface of the agar.

The Chitinoid Annulus The chitinoid annulus does not turn blue in I-KI and can readily be stained with blue ink. Carter's Midnight Blue ink is very effective. Examples for class use are *Diaporthe* spp. and *Lasiosphaeria* spp. The latter may show variously ornamented spheres just below the annulus. Chadefaud (1960) separates three orders with amyloid annuli (Diatrypales, Hyponectriales and Xylariales) from three orders with chitinoid annuli (Diaporthales, Nectriales and Sordariales).

See also Shoemaker, 1964.

STIMULUS-RESPONSE SYSTEMS OF *PHYCOMYCES BLAKESLEEANUS*

The aerial sporangiophore of the fungus *Phycomyces blakesleeanus* is a large, rapidly growing, multinucleated cell that responds to a wide variety of stimuli. For this reason it is of interest to persons working on the physiology of stimulus-response systems. Since it is a single cell that can respond to stimuli in ways analogous to complex animal systems, it is assumed that an understanding of the processes in such a simple system may better enable us to understand the mechanisms of a more complex one. *Phycomyces* cells also have the advantage that they are readily grown in the laboratory. They also can be studied with simple equipment and respond rapidly to stimuli with large observable growth responses.

Life Cycle

The cells are cultured vegetatively from a stock spore suspension. A sketch of the life cycle is shown in Figure 28, and the known stimulus-sensitive stages are marked with a "+". Spores are placed upon a sterile growth medium such as potato slices and kept covered and in a cool, dimly-lit container in which the humidity is kept high (about 65°F, light from a 7.5-watt incandescent refrigerator bulb approximately a foot away, and humidity greater than 80%). Within two days a good mycelial growth should cover the media and by the fourth day small Stage-I sporangiophores should be forming on the surface. About 12 hours later (see exception under trapped air experiment below), a small yellow sphere (the sporangium) begins to form as elongation growth stops. This sphere contains maturing vegetative spores. The sporangium darkens and in Stage IV elongation growth resumes. Mature sporangiophores that are most sensitive have a black sporangium, are about 2 cm in length, and are elongating about 1/8" per hour. The sporangium is about 0.5 mm in diameter, and the very nearly transparent sporangiophore is about 0.1 mm in diameter. Mature cells can reach 6" in length.

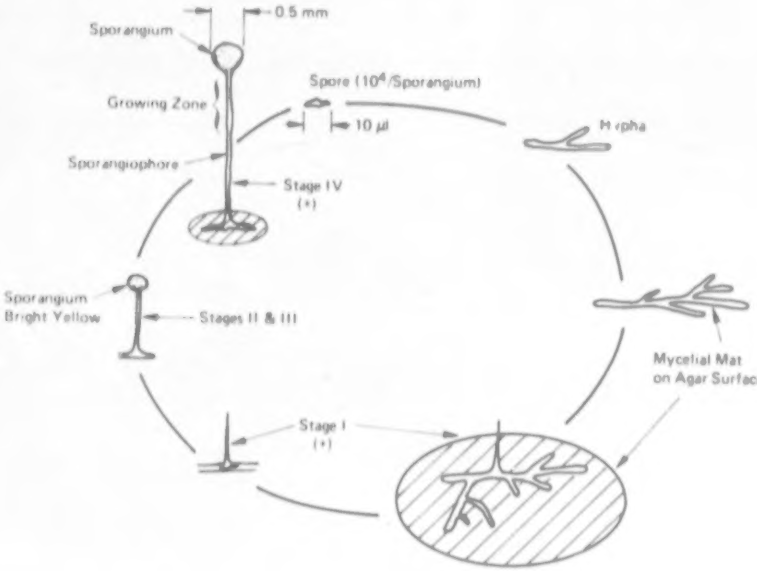
0.5 mm
Sporangium
Spore (10^4/Sporangium)
Growing Zone
10 µl
Hypha
Sporangiophore
Stage IV
(+)
Sporangium
Bright Yellow
Stages II & III
Mycelial Mat
on Agar Surface
Stage I
(+)

FIGURE 28 *Sketch of vegetative life cycle of Phycomyces blakesleeanus.*

Spore Suspension

A spore suspension for maintaining cultures may be made by carefully plucking a mature sporangiophore with your fingers and placing the sporangium end into a small vial with 1 ml of sterile distilled water in it. With a sterile glass rod, crush the sporangium. This grav suspension should have approximately 10^4 spores/ml. It will store well for about 2 weeks in the refrigerator (20°C) as a stock suspension.

A better suspension can be made by allowing mature sporangiophores to grow against the lid of a deep Petri dish or storage jar. Spin the cover to break as many sporangia as possible and then rinse the broken sporangia from the lid with a few ml of sterile distilled water, scraping off spores with a small glass stirring rod. Such a spore suspension can be counted under a 44X objective with a haemocytometer. A suspension containing greater than 10^7 spores/ml will keep in the refrigerator for at least 6 months or longer. Such a suspension may be air dried by placing a small steril vial filled with spores centrifuged to the bottom at low speed in a clinical centrifuge and allowing them to dry above a desiccant. Dried spores have been kept for several years with full viability.

Inoculation

Dilute out the spores with sterile distilled water so that 1 ml will contain about 500 spores. Be sure to shake the suspensions well when diluting, since they settle out rapidly. Just after the final dilution is made, place the diluted suspension in a water bath at 45°C for about 15 min. This heat shock assures that a high percentage of the spores will germinate and grow. Then with a sterile eyedropper or pipette place 0.01 ml (one small drop) on the growth media at a rate of one drop per each square centimeter of surface.

If you do not wish to use spores, or your spore suspension has become contaminated with other fungi, simply cut a small square of the mycelium from near the rapidly-growing edge on an agar plate and place on a new plate.

Within 24 hours the mycelium should nearly cover the surface of a 10-cm plate. Mycelium can usually outgrow most normal contaminants in this manner to obtain a good new stock.

Growth Media

It is advisable to keep the growth media under sterile conditions for about 48 hours after inoculation, to prevent contamination by other organisms. By this time the *Phycomyces* mycelium should be well established and does not have to be handled aseptically.

Autoclaved 1/4"-thick potato slices or autoclaved English muffins in deep, 10-cm Petri dishes with water added work well. A 5% by weight potato glucose agar in water makes a good medium. Occasionally, commercial potato glucose agar does not contain sufficient thiamine for good growth. One ml of a stock thiamine solution of 1 mg/10 ml of water added per liter of agar will give good growth. A convenient culture method is to fill 1-cm shell vials 3/4 full of agar and keep sterile in storage jars until good mycelial growth has been established.

If a liquid culture is desired for measuring mycelial growth rates, or for demonstrating thiamine deficiencies, an Odegård (1952) medium may be simply made:

1.	$CaSO_4 \cdot 2H_2O$	344 mg
2.	$Fe_2(SO_4)_3 \cdot 9H_2O$	162 mg
3.	$ZnSO_4 \cdot 7H_2O$	194 mg
4.	Thiamine HCl	5 mg

Dissolve above compounds in 100 ml distilled water. From this solution take 1 ml and add to the compounds below:

1.	Glucose	30.0 g
2.	Asparagine	3.5 g
3.	KH_2PO_4	1.0 g
4.	$MgSO_4 \cdot 7H_2O$	0.5 g

Dissolve in 1 liter of distilled water.

Growth Chamber

A simple cabinet (Figure 29) for obtaining uniform growth may be made by using a plastic cake cover or cardboard box lined with plastic wrap. A small white light is placed above the top (7.5-watt refrigerator lamp) and an open dish of aqueous saturated ammonium chloride inside to maintain the humidity. Plastic gun-shell loading racks make convenient holders for shell vials, or these could be made out of lucite. If wood is used, be sure to coat it well with paraffin to decrease vapors that inhibit *Phycomyces* growth.

Observational Set-up

Any low-power microscope that has a long focal length will work well (see Figure 30). A regular compound instrument that has a 2X objective lens (available from Edmund Scientific Company, Barrington, New Jersey 08007) inserted and placed horizontally can be used for measuring responses. Remove the mirror and place a red safelight about a foot away. The safelight can be made from an incandescent lamp with a number of layers of red cellophane over a small hole in a box placed over it. To check that it really is a safelight, place a sporangiophore about 3 feet away from the safelight in a completely dark room and leave it on overnight. If the sporangiophore bends toward your safelight, add a couple more sheets of red cellophane.

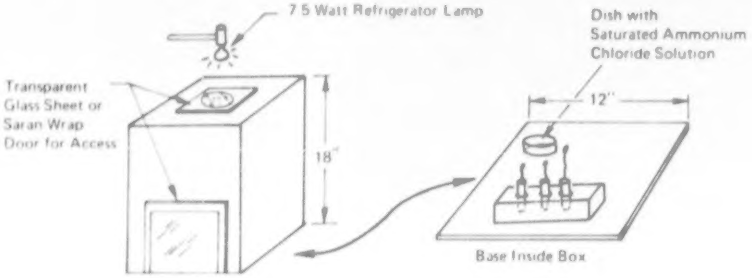
7.5 Watt Refrigerator Lamp
Dish with Saturated Ammonium Chloride Solution
Transparent Glass Sheet or Saran Wrap Door for Access
12"
18"
Base Inside Box

FIGURE 29 *Growth chamber.*

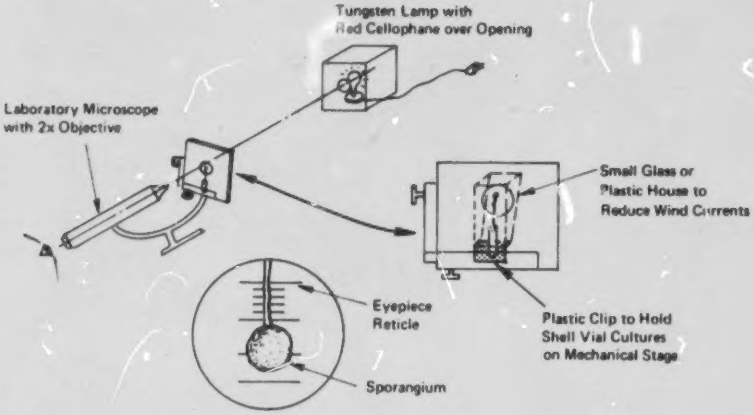
Tungsten Lamp with
Red Cellophane over Opening
Laboratory Microscope
with 2x Objective
Small Glass or
Plastic House to
Reduce Wind Currents
Eyepiece
Reticle
Plastic Clip to Hold
Shell Vial Cultures
on Mechanical Stage
Sporangium

FIGURE 30 *Microscope for observation.*

For measuring growth responses you will need a reticle in the eyepiece of your microscope (also available from Edmund Scientific) and a watch with a sweep second hand. Line up the reticle so it is at right angles to the long axis of the sporangiophore and take measurements each minute.

For making angular measurements use a cross-hair reticle or a center line of the growth reticle. Tape or glue a paper-clip to the eyepiece rim holding the reticle and glue a plastic protractor that has been cut near the center on the barrel of the microscope. Bend the paper-clip pointer to read 90° and in such a way that it moves parallel to the protractor.

Blue Light Source

A small electrical mini-box with a tungsten lamp and several layers of blue cellophane are sufficient. A much larger number of experiments can be done if a variac is available for adjusting the lamp voltage (see Figure 31).

If a blue light source is used, relative intensity measurements may be made using a photographic light meter or any photomultiplier detector if available. For very low light intensities Kodak Wratten Neutral Density filters may be placed over the blue source. An absolute calibration of the light source may be made by determining the maximum intensity for which the phototropic response to a single unilateral stimulus disappears. This intensity is approximately 1000 microwatts/cm^2.

Suggested Experiments

Keeping in mind the fact that *Phycomyces* sporangiophores can change their sensitivity by adapting to the ambient light intensity, it is a good idea to let sporangiophores adapt for at least 30 min to the ambient light intensity

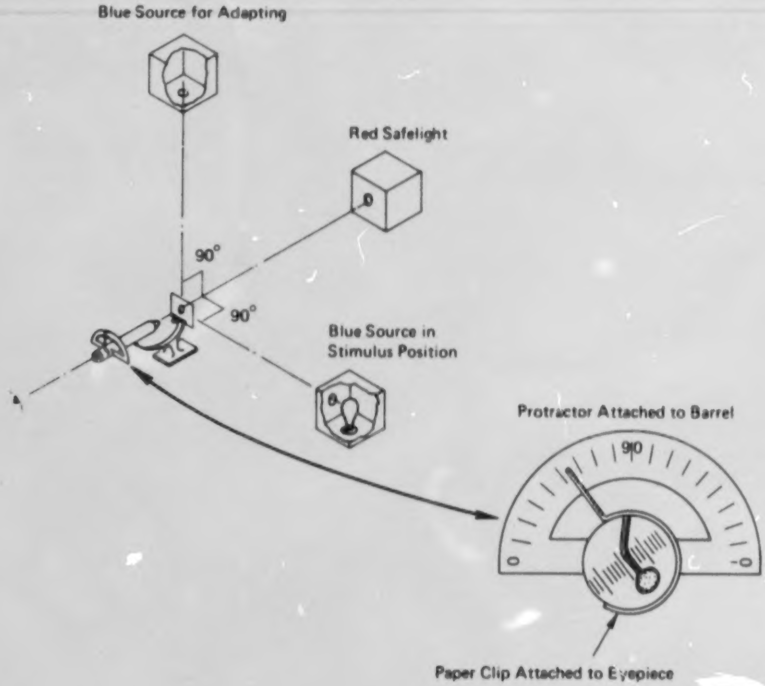
Blue Source for Adapting
Red Safelight
90°
90°
Blue Source in Stimulus Position
Protractor Attached to Barrel
90
0
0
Paper Clip Attached to Eyepiece

FIGURE 31 *Eyepiece goniometer made from protractor and paper-clip; positions for blue source indicated.*

before beginning an experiment. Another good idea is to cover the sporangiophores with a small plastic box to keep wind currents at a minimum.

Phototropism Let the sporangiophore adapt to a low intensity blue light coming from above, or from the side if a fast (at least 2 rpm) turntable is available to rotate the sporangiophore about its own axis, for 30 min. At time zero, move the blue source so the light is coming from one side (or simply stop rotation if using turntable). Measure the angle of bend at one-minute intervals for 20 min. Plot angle of bend vs. time. (See Figure 32.)

Lens Experiment Place several sporangiophores in a plastic container with flat sides. Fill the container with paraffin oil. A metal weight may be necessary to hold the sporangiophore vials down. Expose to blue light from one side. As a control, repeat without the paraffin oil. (See Figure 33.)

Light-growth Response This type of experiment requires a little more elaborate set-up but is well worth the effort because of the large number of experiments that can be done. A bilateral set-up may be constructed with a couple of sheets of masonite or thin aluminum, two

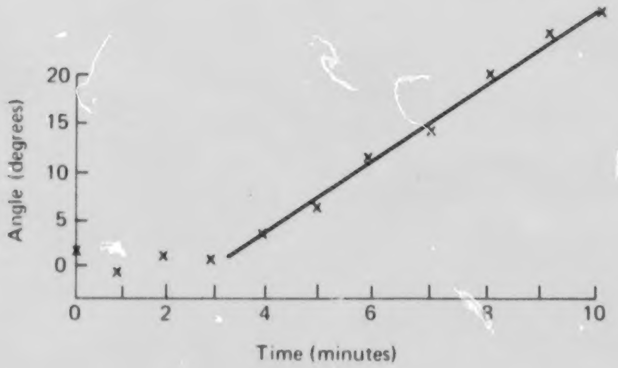

FIGURE 32 *Phototropic experiment.*

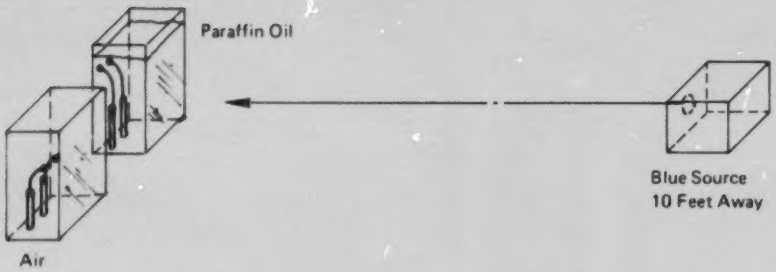

FIGURE 33. *Paraffin oil experiment.*

mirrors and a beam splitter. These are mounted as shown in Figure 34 and clamped to the microscope stage. The left and right beam intensities may be balanced by inserting glass cover slips at point A or B. Use a sporangiophore to determine when equal intensities are reached. Each cover slip reduces the intensity by about 10%. Adapt the sporangiophore to a low intensity of blue light and at time zero give a pulse of bright light for one minute. Follow the growth rate for 15 minutes, at one minute intervals. Plot growth rate vs. time.

Helix Formation If a slow turntable or synchronous motor is available (1/4 rph), a frozen-wave helix may be formed by placing sporangiophores on the turntable and exposing them to continuous low intensity blue light. After 24 hours a good helix should have formed in the sporangiophore. (See Figure 35.)

Geotropic Response Geotropic responses may be demonstrated by placing the sporangiophores in a horizontal position and measuring the final angle of bend after many hours. Care must be taken to eliminate stray light, which will produce phototropic curvatures.

Cytoplasmic Streaming If a sporangiophore is carefully plucked from the agar and placed on a cover slip with a couple of drops of vaseline holding it in place, and so that the sporangium extends out beyond the cover slip, it can be inverted on a couple of cover slip spacers and

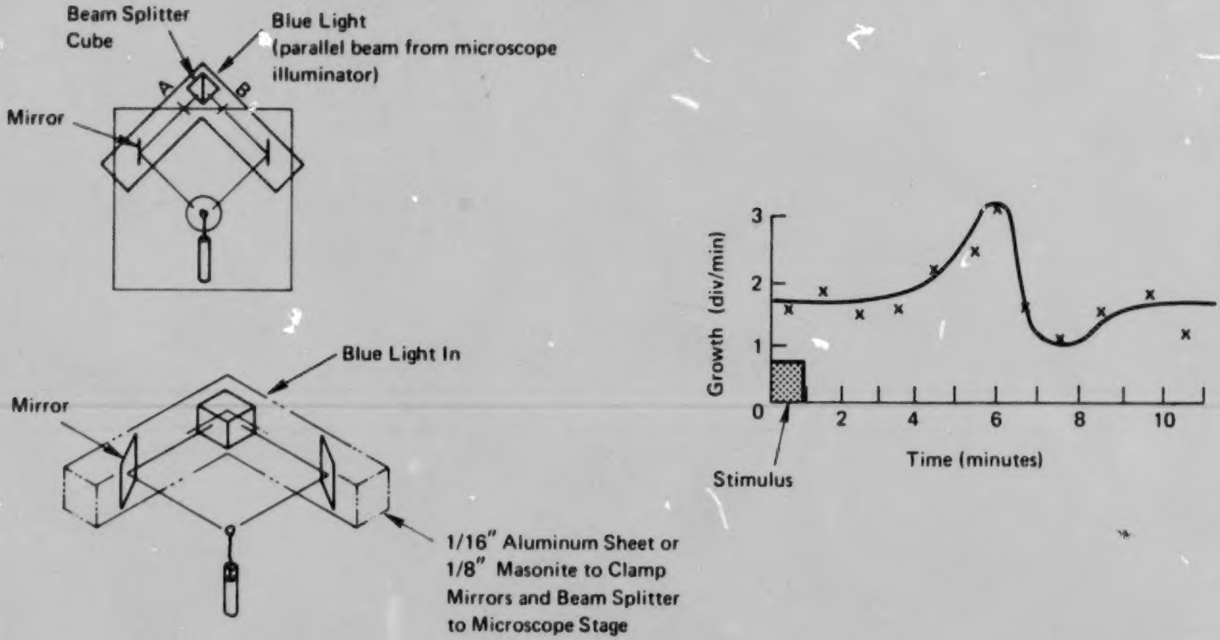
Beam Splitter Cube
Blue Light (parallel beam from microscope illuminator)
A
B
Mirror
Blue Light In
Mirror
1/16" Aluminum Sheet or 1/8" Masonite to Clamp Mirrors and Beam Splitter to Microscope Stage
Growth (div/min)
3
2
1
0
2
4
6
8
10
Time (minutes)
Stimulus

FIGURE 34 *Experimental set-up and typical growth response data.*

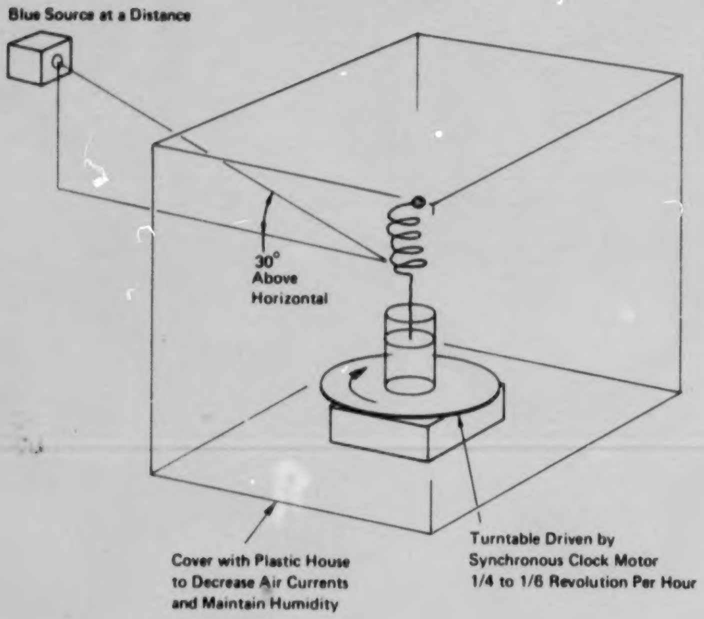
Blue Source at a Distance
30° Above Horizontal
Cover with Plastic House to Decrease Air Currents and Maintain Humidity
Turntable Driven by Synchronous Clock Motor 1/4 to 1/6 Revolution Per Hour

FIGURE 35 *Helix formation.*

the space filled with a few drops of water. Be sure to cover the basal end with a drop of water. Then focus down with a compound microscope, using an oil immersion lens, and observe cytoplasmic streaming. Measure streaming rates in up and down directions. Little is known about the factors controlling streaming and it is postulated that a cross-section of the sporangiophore would appear as a corrugated structure with particles streaming up and down, side by side in very nearly the same plane of focus. (See Figure 36.)

Avoidance Response If a barrier is brought near to a sporangiophore growing zone without touching it, the sporangiophore will grow away from the barrier. The barrier must be brought to within two cell diameters in order to get a good response. Be sure not to hit the sporangium. Determine minimum exposure time to produce an avoidance response. (See Figure 37.)

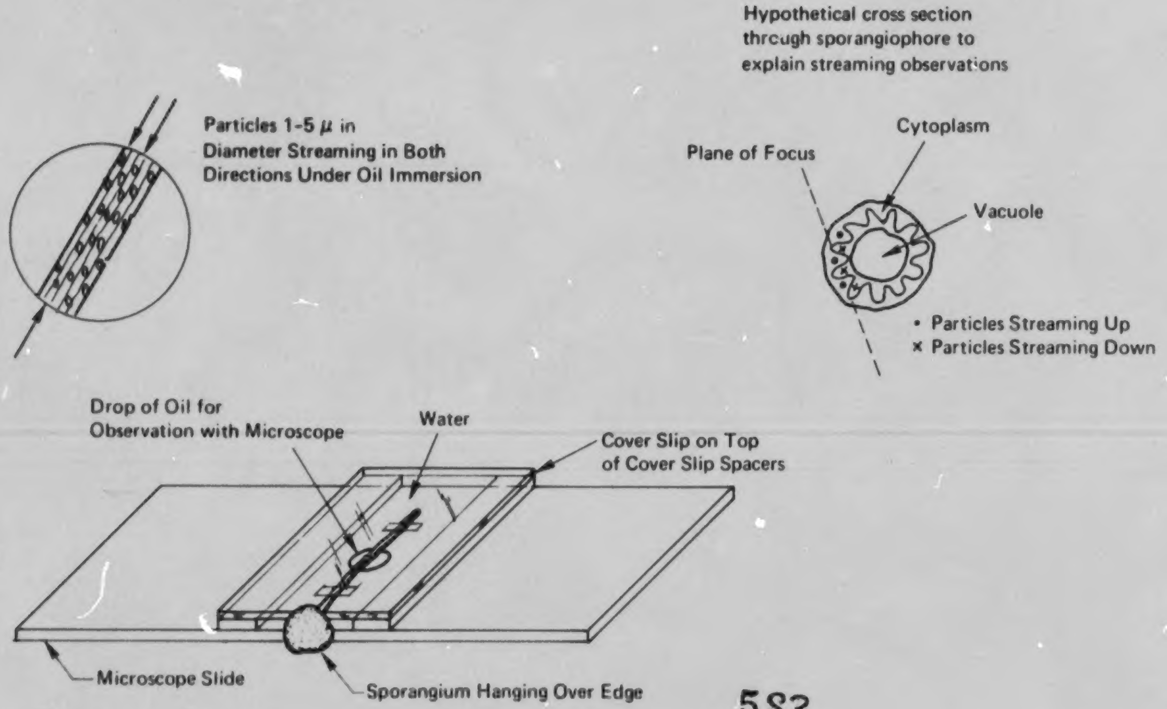
Hypothetical cross section
through sporangiophore to
explain streaming observations
Particles 1-5 μ in
Diameter Streaming in Both
Directions Under Oil Immersion
Cytoplasm
Plane of Focus
Vacuole
• Particles Streaming Up
× Particles Streaming Down
Drop of Oil for
Observation with Microscope
Water
Cover Slip on Top
of Cover Slip Spacers
Microscope Slide
Sporangium Hanging Over Edge

FIGURE 36 *Cytoplasmic streaming.*

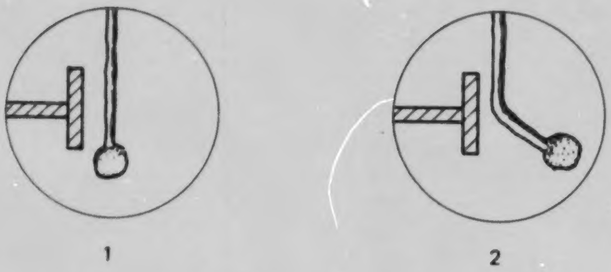

FIGURE 37 *Avoidance response: 1 = at outset; 2 = during response.*

Responses to Mechanical Stimuli Mechanical stimuli may be given by pushing against a sporangium with a long thin glass fiber. If a small drop of oil is placed on the sporangium, the fiber will stick and a pull can be given. If you are very patient, a small hook (nichrome wire, Dennison and Roth, 1967) may be made and weights may be hung on the sporangiophore. (See Figure 38.)

Trapped-air Experiment It has been observed that if Stage-I sporangiophores are placed in a closed small

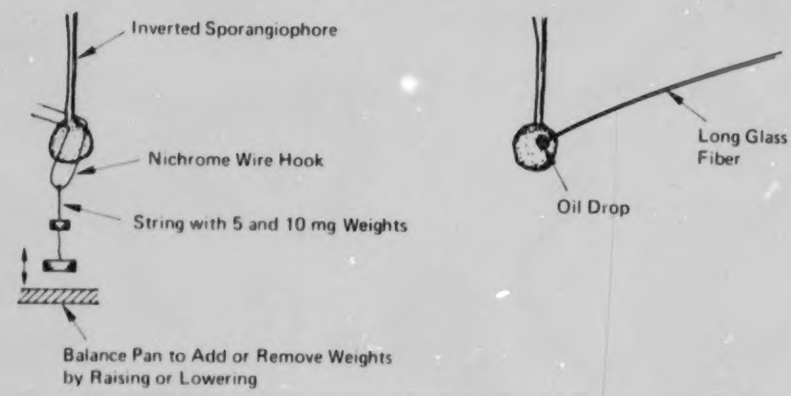
Inverted Sporangiophore
Nichrome Wire Hook
String with 5 and 10 mg Weights
Balance Pan to Add or Remove Weights by Raising or Lowering
Long Glass Fiber
Oil Drop

FIGURE 38 *Set-up for response to mechanical stimuli.*

container with at least one Stage-IV sporangiophore present, then sporangia do not form on the young sporangiophores. In fact, they will grow up to 15 cm long without sporangia ever forming. If the cover is lifted off momentarily so that fresh air is admitted, sporangia begin to form within a few hours. The nature of this gas is unknown. (See Figure 39.)

Plucked Sporangiophore It has been known for some time that sporangiophores apparently contain everything required for their growth except water. If a young Stage-IV or even a Stage-I sporangiophore is gently plucked and stuck to the side of a vial with vaseline so that the basal end is immersed in water, it will continue to grow and respond for many hours. (See Figure 40.)

Demonstration of the Growing Zone The wall in the growing zone that is about 3 mm long and just below the sporangium is thinner and less rigid than the mature wall. Apparently, the internal pressure of the vacuole keeps this portion of the cell rigid, just as a balloon is blown up. If a sporangiophore is plucked gently and held lightly just above the basal plug, a pair of scissors can be used to cut off the base. Immediately the cell will collapse in the growing zone region. If pressure is lightly applied with the fingers, the internal pressure can be restored and the growing zone will

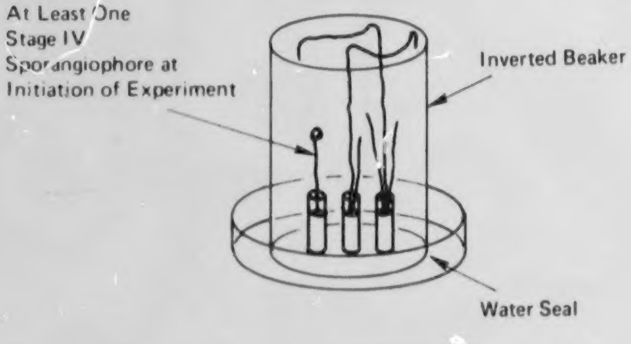
At Least One Stage IV Sporangiophore at Initiation of Experiment
Inverted Beaker
Water Seal

FIGURE 39 *Trapped-air experiment.*

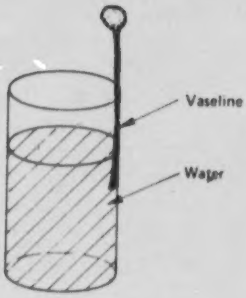

FIGURE 40 *Plucked sporangiophore.*

straighten out. All of these operations may be seen with the naked eye and are quite startling as proof that the sporangiophore is indeed a single cell with no cross walls. Stage-IV sporangiophores about 5 cm in length are best for this demonstration.

Requirement for Thiamine Classically, *Phycomyces* has been used as a microbiological assay for thiamine. Small flasks with 50 ml of Odegård medium can be inoculated with 25 spores each. Aliquots of thiamine ranging from 0 to 150 μg per flask can be added and the growth of the mycelium measured either as diameter of cultures or fresh or dry weight of mycelia produced. Cultures should be assayed after 5 to 7 days growth. A linear plot of growth (weight of mycelial pad) as a function of thiamine added should be obtained over this range.

Precautions

The above experiments are suggested because they require a minimum of equipment for measuring interesting cellular responses to external stimuli. They do require, however, varying degrees of skill by the experimenter. It is suggested that a phototropic response be the first one attempted since it is the most fool-proof one. If this experiment does not work, then one of the following things may be wrong:

- The blue light source may be too bright. Place a piece of onion-skin typing paper over the blue source.
- There may be light leaks in the room where you are doing experiments. If this is a problem, place the entire experimental set-up in a large cardboard box with black cloth to keep out stray light.
- The red safelight may not be safe. Test as outlined above.
- The room for experiments may be too warm. If the temperature is above 80°F, sporangiophores grow very poorly if at all.
- The room for experiments may be too dry. Especially in the winter-time, if the humidity is below 40%, sporangiophores will not grow or respond well. If this is suspected, place a couple of strips of moistened paper in the plastic house used to keep wind currents to a minimum.
- Be sure to select good mature sporangiophores that are growing well. If these conditions are met, and they can be easily reached with a minimum of effort, you should have little difficulty.

See also Shropshire, 1963; Bergman *et al.*, 1969.

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APPENDICES

Biological Materials

QUARANTINE AND SHIPMENT

Users of this *Guidebook* are reminded that "The Federal Plant Pest Act of May 23, 1957, prohibits the movement of any plant pest from a foreign country into or through the United States, or interstate, unless such movement is authorized under a general or specific permit issued by this Department (USDA)."

Generally, the necessary permission to procure cultures of microorganisms for use in teaching and research is given promptly, but as long as the laws remain as they are now, there is no alternative to applying for formal permission. For further guidance in this matter, the rules and regulations of the Plant Protection and Quarantine Programs are reproduced below.

> ### Instructions to Importers or Receivers of Cultures
>
> The Federal Plant Pest Act of May 23, 1957, prohibits the movement of any plant pest from a foreign country into or through the United States, or interstate, unless such movement is authorized under a general or specific permit issued by this Department. Included in the meaning of the term "plant pest" are nematodes, bacteria, fungi, other parasitic plants or reproductive parts thereof, viruses or any similar organisms or infectious substances which can cause disease or damage to plants or plant products.

It is suggested that persons contemplating the importation or interstate movement of any plant pest as defined above (including cultures thereof), or of nonpathogenic organisms, follow the appropriate procedure outlined below:

I. Importation or Interstate Movement of Plant Pathogenic Organisms

a. Apply, in advance of importation or movement, to the Plant Protection and Quarantine Programs Animal and Plant Health Inspection Service, USDA, FCB-1, Hyattsville, Maryland 20782, for a permit and official shipping labels to authorize entry or movement of the material concerned. In applying, furnish by letter the information called for in PPQ Form 26; you may request a supply of the forms for your convenience in applying for permits. (See III for exceptions.)

b. Secure advance approval from the state plant quarantine official of the state of destination. Evidence of such approval may be furnished in Section B of the application form or may be submitted separately by letter or other written communication.

c. Instruct foreign shippers to forward cultures or organisms by either air or surface mail, using the official green and yellow labels furnished with the permit, such labels to be used in accordance with the instructions appearing on the reverse side thereof. For interstate shipments a different type label will be furnished for use on shipments forwarded by any means (mail, express, freight, etc.).

II. Importation or Interstate Movement or Nonpathogenic Organisms

a. Inform the Plant Protection and Quarantine Programs, in advance, of the names of the organisms concerned and the number of cultures to be imported or moved interstate so that official labels may be furnished as a courtesy to effect prompt delivery of the parcels. The application for permit form may be used for this purpose.

b. Instruct shippers to forward the parcels and to use the official labels supplied by the Plant Protection and Quarantine Programs. This is important since inspectors at ports of entry, Post Offices, and other shipping points cannot always be expected to be able to distinguish between harmful and innocuous organisms. Shipments moving without labels may be subject to delay in delivery while the pest status of the organisms concerned is being determined.

III. Regulations Enforced by Other Federal Agencies

Importation or Interstate Movement of Human or Animal Pathogens or Vectors

Persons interested in human or animal pathogens should address requests for permits as follows:

For Human Pathogens:
Foreign Quarantine Program
National Communicable Disease Center
Atlanta, Georgia 30333

For Animal Pathogens:
Veterinary Services
APHIS, USDA, Room 813
Federal Center Building
Hyattsville, Maryland 20782

Importation of Soil Samples into the United States

Foreign soils can be the means of introducing many serious plant pests into the United States. Surface soil is especially notorious as a carrier of various pests, including insects, fungi, bacteria, nematodes, and virus diseases. For that reason the importation of soil is restricted under regulations enforced by the Plant Protection and Quarantine Programs of the U. S. Department of Agriculture.

Soil samples may be imported into the United States under permit and subject to such treatment or other safeguard conditions as may be necessary to prevent the introduction and dissemination of injurious plant pests. Soil samples are usually given a dry heat or steam sterilization treatment upon arrival in the United States and before release to the importer. Small samples can usually be treated at one of the special inspection stations of the Plant Protection and Quarantine Programs without cost to the importer.

In order to import soil samples without treatment, special arrangements must be made for storage, use, and final disposition at destination in such a manner that there will be no danger of plant pest escape.

Persons wishing to receive soil samples from foreign countries should first obtain a permit authorizing movement of the samples. Any necessary instructions on import procedures will be furnished with the permit. The application for a permit should be addressed to the Permit Office, Plant Protection and Quarantine Programs, U.S. Department of Agriculture, 209 River Street, Hoboken, New Jersey 07030.

The *Guidebook* has as one of its central objectives to encourage teachers to secure their own material in the field, but in many instances it will be necessary to resort to individuals or institutions as sources of the desired cultures.

At several points in the *Guidebook*, and in a following section, specific sources of fungi are cited in connection with a given study. The full list of contributors is to be found at the back of the volume. In most cases these persons will be willing to supply material directly related to their specialty, or suggest ways in which it can be obtained. However, it must be emphasized that there is no implicit commitment on the part of those who contributed to the *Guidebook* to supply cultures. When they do so, it must be recognized as a gesture in support of mycology teaching, not response to an obligation.

In a limited way, Federal and State agricultural experiment stations will also be suitable sources of teaching material, especially as it relates to pathogenic fungi.

CULTURE COLLECTIONS*

A substantial portion of the organisms cited in the *Guidebook* are available from major culture collections, perhaps chiefly the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852. Contributors to the *Guidebook* have been urged to deposit with one or more of these institutions any organisms cited in their material that are particularly useful or necessary for successful laboratory work. As more strains are made available, the lists of holdings will change; for this reason, no listing of species is included here, although certain special information is incorporated in the immediately following section of this Appendix. Each instructor should, when the occasion arises to procure cultures by purchase, contact the culture collections most readily available to him for up-to-date information.

The first culture collections were established about 1900. Several have survived to the present time. Today there are literally hundreds of small collections scattered in government, university, and industrial laboratories all over the world. Actually, only a few are permanent and at all comprehensive in the kinds of microorganisms maintained. Some collections may be very restricted in their scope but may be the most complete collection on a single genus available anywhere. Usually they represent the life work of a specialist and often they are lost upon his retirement. Most of these small collections were established for research and teaching purposes and are not easily located.

On the other hand, there are larger, more comprehensive collections that have formal status. The ones maintained by industry are large but generally inaccessible to the public. There are also comprehensive collections located in universities and government laboratories and at least one, The American Type Culture Collection (U.S.), that is a private nonprofit organization, supported by both government and private grants, including ones from scientific societies. Part of its income, as well as support for several other collections, is derived from the sale of cultures and other culture services.

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*Much of the information in this section was assembled by Dr. Clifford Hesseltine.

In the following list of major culture collections, the approximate holdings in strains are given to the extent the information is available.

Center for Forest Mycology Research Forest Products Laboratory Madison, Wisconsin 53705	4,500
Prairie Regional Laboratory Saskatoon, Saskatchewan Canada	
Laboratoire de Cryptogamie Museum National d'Histoire Naturelle Paris, France	
Institute of Applied Microbiology University of Tokyo Bunkyo-ku, Tokyo, Japan	2,120
Culture Collection Institute for Fermentation (IFO) 4-54, Jusonishinocho, Higashiyodogawaku Osaka, Japan	6,740
Centraalbureau voor Schimmelcultures (CBS) Baarn, The Netherlands	12,700
Culture Collection Laboratory of Microbiology Technical University Julianalaan 67A Delft, The Netherlands	3,000
Council for Scientific and Industrial Research Pretoria, South Africa	

The Commonwealth Mycological Institute (CMI) 5,000
The Ministry of Technology
Ferry Land, Kew, Surrey, England
United Kingdom

British National Collection of Yeast Cultures
Brewing Industry Research Foundation
Nutfield, Surrey, England
United Kingdom

Culture Collection of Algae and Protozoa
Cambridge University
Cambridge, England
United Kingdom

National Collection of Industrial Bacteria 3,600
Ministry of Technology
P.O. Box 31
135 Abbey Road
Aberdeen, Scotland
United Kingdom

American Type Culture Collection (ATCC) 11,400
12301 Parklawn Drive
Rockville, Maryland 20852

ARS Culture Collection 17,387
Northern Marketing and Nutrition
  Research Division
Agricultural Research Service
U.S. Department of Agriculture
1815 North University Street
Peoria, Illinois 61604

Culture Collection of the U.S. Army Materiel Command
U.S. Army Natick Laboratories
Natick, Massachusetts 01760

The Culture Collection of Algae
Department of Botany
Indiana University
Bloomington, Indiana 47401

IMRU Collection
Institute of Microbiology
Rutgers, The State University
New Brunswick, New Jersey 08903

Fungal Genetics Stock Center
Humbolt State College
Arcata, California 95521

All-Union Collection of Type Cultures 4,500
Department of Type Cultures
Institute of Microbiology
U.S.S.R. Academy of Sciences
Profsouznaya Str., 7a
Moscow, B-133, U.S.S.R.

Institute of New Antibiotics
Moscow, U.S.S.R.

Japanese Federation of Culture Collections

70,947

SPECIAL CULTURE INFORMATION

It is common experience in working with fungus cultures to find that success or failure in an experiment or demonstration depends upon having a particular strain of the organism or having a culture that has not over a period of time lost the ability to perform in the desired manner. This situation is so pervasive that the mycology instructor must learn to live with it and to maintain a careful surveillance of his stocks with this very difficulty in mind.

Because so very much depends on the particular circumstances in a given laboratory or the particular organism in question, the *Guidebook* does not attempt to indicate particular strains of most organisms cited. The user will, with experience, come to make the necessary allowances and adjustments within the context of his own facilities. In those sections where the source of the organism is explicitly stated or where it is essential to use a given numbered strain--at least at the outset--the additional information is provided below. The sections listed will be found to occur in the same order as they appear in the body of the *Guidebook*; where the contributor's name is necessary for reference, it is provided in parentheses following the title of the section.

Cellular Slime Molds (K. B. Raper)

Dictyostelium discoideum	NC-4
Dictyostelium mucoroides	WS-142
Dictyostelium purpureum	WS-321
Polysphondylium violaceum	V-9
Polysphondylium pallidum	WS-320
Escherichia coli	B-281
Enterobacter aerogenes	B-900
Bacillus megaterium	B-160
Serratia marcescens	B-175

Labyrinthulales (David R. Porter)

Cultures of *Labyrinthula*, including isolate L 69-18.

Zoosporic Phycomycetes (Ralph Emerson)

Rozella allomycis Foust (47-54)
Allomyces arbuscula Butler (Philippine Islands 1)
Rhizophydium sphaerotheca Zopf (55-1)
Nowakowskiella elegans (Nowak.) Schroeter (50-1)
Blastocladiella simplex Matthews (48-8)
Allomyces (*Euallomyces*) *macrogynus* (Emerson) Emerson and Wilson (Burma 3)
Saprolegnia sp. (47-15)
Achlya sp. (48-2)
Achlya ambisexualis Raper (50-13 ♂ x 50-15 ♀)
Aphanomyces sp. (53-15b)
Apodachlya sp. (47-17)
Pythium spp. (55-2), (47-11)

Pythium aphanidermatum (Edson) Fitzpatrick (51-18)
Pythium oligandrum Drechsler (51-20)
Phytophthora spp. (47-1)
Phytophthora drechsleri Tucker (51-11 ♂ and 51-12 ♀)

Hemiascomycetes (Protomycetaceae--L. R. G. Valadon)

Cultures of *Protomyces inundatus* may be obtained from the Northern Regional Research Laboratories, Peoria, Ill., where the (+) strain has the number NRRL Y-6802 and the (-) strain NRRL Y-6803, and also from the Commonwealth Mycological Institute, Kew, Surrey, where the diploids are under numbers 96, 163; 96, 197; and 96, 198.

Plectomycetes (Eurotiales)

Representative genera, with conidial stage where known, and ATCC culture number, are listed below:

Byssochlamys nivea (Paecilomyces)	ATCC	#12550
Gymnoascus reessii	"	13485
Shanorella spirotricha	"	12594
Carpentales javanicum (Penicillium)	"	9099
Emericella variecolor (Aspergillus)	"	14883
Eurotium repens (Aspergillus)	"	9294
Kernia spirotricha	"	12128
Monascus purpureus	"	6405
Sartorya fumigata (Aspergillus)	"	1020
Talaromyces vermiculatus (Penicillium)	"	10511
Thielavia sepedonium (Sepedonium)	"	9787

Discomycetes

Lambertella -- culture 53-12, Microgarden, Department of Botany, University of California, Berkeley.
Peziza quelepidotia -- from the Mycology Laboratory of E. S. Beneke, Department of Botany & Plant Pathology, Michigan State University, East Lansing.

Fungi Imperfecti (Aspergillus and Penicillium--K. B. Raper)

Aspergillus clavatus	WB-2254
Aspergillus fumigatus	WB-4537
Aspergillus nidulans	WB-189
Aspergillus terreus	WB-225
Aspergillus flavipes	WB-1959
Aspergillus niger	WB-326
Aspergillus versicolor	WB-239
Aspergillus ustus	WB-1974
Aspergillus flavus	WB-1957
Aspergillus candidus	WB-303
Aspergillus repens	WB-13
Aspergillus ornatus	WB-2256
Aspergillus janus	WB-1787
Aspergillus giganteus	WB-10
Penicillium javanicum	WB-707
Penicillium sclerotiorum	WB-20704
Penicillium frequentans	WB-1915
Penicillium chrysogenum	NRRL-1951 = ATCC-9480
Penicillium digitatum	WB-1202
Penicillium roquefortii	WB-849; WB-5180
Penicillium camembertii	WB-877
Penicillium caseicola	WB-874
Penicillium italicum	WB-1900
Penicillium expansum	WB-973
Penicillium claviforme	WB-2031
Penicillium isariiforme	WB-4202
Penicillium purpurogenum	WB-1214
Penicillium vermiculatum	NRRL-2098

Fungi Pathogenic to Insects

Entomophthora coronata	ATCC-10051
Beauveria bassiana	ATCC-9835

Thermophilic Fungi

Mucor miehei	ATCC-16457
Mucor pusillus	ATCC-16458 and 16459
Chaetomium thermophile var *coprophile*	ATCC-16451
Humicola lanuginosa	ATCC-16455

Osmophilic Fungi

Aspergillus halophilicus	WB-4679
Aspergillus ruber	WB-70, WB-52
Aspergillus medius	WB-124
Aspergillus carnoyi	WB-126
Aspergillus amstelodami	WB-90
Aspergillus arenarius	WB-5012
Aspergillus penicilloides	WB-4550

Sexual Agglutination in Yeasts

Saccharomyces kluyveri	NRRL Y-4288-3; Y-4288-26
Hansenula wingei	NRRL Y-2340-5; Y-2340-21

Heterokaryosis in Aspergillus

Aspergillus carbonarius	WB-67 (brown mutant)
Aspergillus fumigatus	WB-1173 (wild type)
Aspergillus fumigatus	WB-5033 (white mutant)

Fungi Cited

All explicit citations to genera and species of fungi appearing in the *Guidebook* are listed alphabetically below with the page or pages on which the citation is to be found. To the extent possible the names are as they appear in the Index to *Mycologia* published in 1968. Genera and species not appearing in that index are alphabetized in their proper place in the listing.

Absidia (93, 96, 98, 336)
  A. spinosa (98)
Abstoma (386)
Achlya (55, 59, 61, 62, 68)
  A. ambisexualis (68)
Acrospermum compressum (180)
Acrosporium (216, 219)
Actinomyces (347, 349, 350)
Acytostelium (25)
Agaricus abruptibulbus (281)
Agaricus bisporus (294, 305)
Allomyces (60, 62, 72, 80, 83)
  A. arbuscula (65, 81, 83)
  A. macrogynus (67, 81, 83)
Alpova (386)
  A. cinnamomeus (387)
Alternaria (217, 218, 222, 337, 358, 380-82)
  A. consortiale (381)
  A. cucumerina (222, 381)

A. tenuis (338, 381, 466)
A. tenuissima (222)
Amanita (281, 282, 323, 326)
A. bisporigera (279)
A. brunnescens (281)
A. muscaria (279)
A. rubescens (177, 326)
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Amblyosporium (216)
Amoebidium (106, 116, 117, 119)
A. parasiticum (116)
Amorphotheca (225)
Ancylistes (339)
Anisostomula (169)
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A. macrocystis (416)
A. maurilabra (415)
A. melaloma (415, 416)
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Apiosporina (202)
A. collinsii (101)
A. morbosa (178, 202)
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Arthroderma (142, 144, 352)
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A. quadrifidum (142, 352)
A. uncinatum (142, 352)
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A. carbonarius (415, 416)
A. furfuraceus (408)
A. immersus (551)
A. stercorarius (183, 408)

  A. striisporus (365)
- Ascochyta cucumis (203)
- Ascodesmis (187, 188, 407, 409)
- Ascoidea hylecoeti (314)
- Ascophanus (407)
  - A. carneus (410)
- Ascozonus (401, 407)
- Asellaria (114, 119)
- Aspergillus (20, 94, 215, 218, 231-37, 350, 380, 381, 465, 495, 524)
  - A. amstelodami (381, 424)
  - A. arenarius (424)
  - A. candidus (236)
  - A. carbonarius (495, 496)
  - A. clavatus (236, 450)
  - A. flavipes (236)
  - A. flavus (236)
  - A. fumigatus (236, 422, 495, 496)
  - A. giganteus (237, 450)
  - A. glaucus (233, 234, 237, 424)
  - A. halophilicus (237, 424)
  - A. janus (237)
  - A. nidulans (236, 487, 495-97, 499, 500, 502, 504, 520)
  - A. niger (236, 381, 495, 498)
  - A. ornatus (237)
  - A. oryzae (367)
  - A. penicilloides (424)
  - A. repens (237)
  - A. restrictus (424)
  - A. ruber (424)
  - A. terreus (236)
  - A. ustus (236)
  - A. versicolor (236, 424)
- Astraeus hygrometricus (295, 298)
- Aureobasidium (217, 223)
  - A. pullulans (223)
- Auricularia (241, 243, 244, 339)
  - A. auricula-judae (303)
- Auxarthron (142)
  - A. brunneum (146)
  - A. californiense (147)
  - A. zuffianum (147)

- Balansia epichloe (179)
- Balsamia (386)
- Barssia (386)
- Basidiobolus (339, 403, 404)
  - B. meristosporus (403)
  - B. ranarum (339, 403)
- Battarraea (6, 295)
- Beauveria (217, 223, 346)
  - B. bassiana (223, 341, 346)
- Bertia moriformis (179)
- Bipolaris (217, 223, 224)
  - B. sorokiniana (224)
- Bispora (217)
- Blakeslea (95)
- Blastocladiella (72, 74, 78)
  - B. britannica (78, 79)
  - B. emersonii (73, 78)
  - B. simplex (66)
- Blastomyces dermatitidis (348, 349)
- Boletinus (294)
- Boletus (282, 323, 326)
  - B. subvelutipes (292)
- Bombardia (405)
- Botryodiplodia theobromae (226)
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  - B. pulchum (224)
- Botryotinia (224)
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  - B. cinerea (224)
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- Caliciopsis (179)
- Calocera (241)
- Calonectria (227)

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  C. fulgens (280)
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  C. gigantea (295)
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  C. silvicola (131)
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  C. cibarius (281)
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  C. javanicum (150, 151)
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  C. coerulescens (153)
  C. fimbriata (153, 337)
  C. minor (153)
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  C. multiannulata (152)
  C. pilifera (152)
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  C. virescens (153)
Ceratostomella fimbriata (464)
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  C. musae (225)
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  C. halima (377)
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  C. convolutum (466)
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  - *C. herbarum* (225, 381)
  - *C. macrocarpum* (381)
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  - *C. purpurea* (178)
- *Clavicorona pyxidata* (280)
- *Clitocybe* (6)
  - *C. illudens* (459, 461)
- *Coccidioides immitis* (347, 348, 354)
- *Cochliobolus* (224)
  - *C. heterostrophus* (224)
- *Coelomomyces* (88, 89)
  - *C. dodgei* (89)
  - *C. lativittatus* (89)
  - *C. psorophorae* (89)
  - *C. punctatus* (89, 90)
- *Coemansia* (95, 402)
- *Cokeromyces* (402)
- *Coleosporium* (255, 256)
- *Coleroa* (202)
  - *C. circinans* (202)
  - *C. robertiana* (202)
  - *C. rubicola* (202)
- *Colletotrichum* (219, 226)
  - *C. gloeosporioides* (226)
  - *C. lagenarium* (226)
  - *C. lindemuthianum* (226)
  - *C. orbiculare* (226)
  - *C. trifolii* (358)

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  C. velutipes (307)
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  C. ephemerus (5)
  C. fimetarius (539)
  C. lagopus (305-7, 539, 543)
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  C. capitata (179)
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   E. coronata (341, 342, 343, 344, 346, 436, 449, 450)
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   E. andropogonis (227)
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Flammulina velutipes (282, 307)
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  F. applanatus (291)
  F. cajanderi (301, 302)
  F. fomentarius (291)
  F. igniarius (302)
  F. pini (302)
  F. pinicola (302)
  F. rimosus (291)
  F. roseus (302)
Fuligo septica (50)
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  F. decemcellulare (227)
  F. fructigena (444)
  F. moniliforme (227)
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  G. autumnalis (280)
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  G. applanatum (302)
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  G. fructicola (172)
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Stains, Reagents, Media

STAINS AND REAGENTS

SR-1 Lactophenol Mounting Medium (Amann)

Phenol (crystals)	20 g	
Lactic acid	20 g	(16 ml)
Glycerol	40 g	(31 ml)
Distilled water	20 g	(20 ml)
Poirrier's (Cotton) Blue	0.05-1.0 g	

Acid Fuchsin may be substituted for the Poirrier's Blue. Water may be omitted.

SR-2 CMC Mounting Medium (as used for Myxomycetes)

Mix thoroughly equal quantities of CMC-9 and distilled water or lactophenol. Mount the specimen in a drop of the medium, cover with cover slip, and heat gently over a small flame. CMC-9AF, which contains a red dye, may be used in the same way as CMC-9 if it is desired to stain the mounted structures. These media may be used for permanent mounts of fruiting bodies, capillitium, and spores of myxomycete species that *do not* contain lime, e.g., *Stemonitis.* CMC-9 is a low-viscosity carboxymethyl cellulose formulation, available through General Biological Supply House (Turtox), 8200 S. Hoyne, Chicago, Ill.

SR-3 *Lactofuchsin Mounting Medium*

Lactic acid (anhydrous)	100 ml
Acid Fuchsin	1 g

SR-4 *Lactic Acid Mounting Medium*

Lactic acid, without additions of any kind, commonly is used for preparing mounts of imperfects, particularly if the specimen has some color of its own. Poirrier's Blue (Cotton Blue) or Acid Fuchsin may be incorporated ad lib., 0.05-1.0 g per 100 ml.

SR-5 *Glycerol-Chloral Hydrate Medium*

Glycerol	100 ml
Chloral hydrate	25 g
Distilled water	25 ml
Acid Fuchsin or Poirrier's Blue	0.1 g

SR-6 *Karo Syrup Mounting Medium*

Karo syrup (white)	100 ml
Water	300 ml
40% Formalin	4 ml

SR-7 *Hoyer's Medium*

Gum arabic	30 g
Chloral hydrate	200 g
Glycerol	16 ml
Distilled water	50 ml

Soak gum arabic in water for 24 hours. Add chloral hydrate and let stand for several days. Then add glycerol. It is advisable to add a trace of chloral hydrate to the gum arabic to prevent bacterial growth during the soaking period.

SR-8 *Patterson's Mounting Fluid*

Potassium acetate, 2% aq.	300 ml
Glycerine	120 ml
Ethyl alcohol (95%)	180 ml

SR-9 Aceto-Orcein Preparation

Dissolve 1 g Gurr's synthetic orcein in 45 ml hot glacial acetic acid; cool. Dilute 1:1 with distilled water, and reflux for 5 min.

SR-10 Azur A Staining Medium
(for conidiophore annellations)

Azur A (CAz-4, Coleman and Bell), prepared as a 1% aqueous solution. (Ref: Shoemaker, R. A. 1964. Stain technology 39:120-121.)

SR-11 Feulgen Stain

Pour 200 ml boiling distilled water over 1 g of Basic Fuchsin and shake. Cool to 50°C, filter into brown or dark-colored stock bottle with ground glass stopper, and add 20 ml 1.0 N hydrochloric acid. Cool further to 25°C and add either 1 g anhydrous sodium bisulfite ($NaHSO_3$) or 1-3 g of potassium metabisulfite ($K_2S_2O_5$). After storage for 24 hours in the dark, the solution should be faintly straw-colored or, better, colorless. If the staining solution has not decolorized sufficiently, add 0.5-1.0 g of decolorizing carbon (coconut charcoal, activated), shake and filter.

SR-12 Janus Green Staining Solution

Prepared as a 0.1% solution in 0.2 M sodium acetate-acetic acid buffer at pH 4.6. (approximately equal volumes of 0.2 M Na-acetate and 0.2 M acetic acid)

SR-13 Phloxine Staining Solution

Phloxine, prepared as a 2% aqueous solution. KOH-Phloxine Staining Method (for Homobasidiomycetes):

- Tease hymenial tissue into a drop of 70% ethyl alcohol; drain off alcohol or allow it to evaporate.
- Add drop of 5% KOH (aqueous solution).
- Follow with a drop of 0.1% aqueous phloxine.

(R. Singer, in "The Agaricales in Modern Taxonomy," notes the common use of 2% phloxine in alcohol.)

SR-14 Melzer's Reagent (Langeron modification)

Chloral hydrate	100 g
Potassium iodide	5 g
Iodine	1.5 g
Distilled water	100 ml

SR-15 Iodine-Alcohol

Add iodine crystals to 70% alcohol to give a light amber color. Used for fixing and lightly staining myxamoebae, pseudoplasmodia, and sorocarps of Acrasiae. Eosin or erythrosin may be added if deeper staining of myxamoebae is desired.

SR-16 I-KI and ZnCl (cellulose stain as used with Acrasiae)

Solution A:	Iodine	1 g
	Potassium iodide	1 g
	Distilled water	100 ml
Solution B:	Zinc chloride	50 g
	Distilled water	25 ml

Kill specimens in Iodine-alcohol, transfer to solution A for a few seconds, then mount in solution B. Cellulose stains blue but color fades quickly.

SR-17 Chloriodide of Zinc (cellulose stain as used with Acrasiae)

Zinc chloride	30 g
Potassium iodide	5 g
Iodine	0.89 g
Distilled water	14 ml

Store in black bottle or away from light. Use after one week. Kill specimen in Iodine-alcohol, mount in water, then draw reagent under cover slip with blotting paper or tissue. Cellulose in sorophore sheath and in walls of stalk cells and spores stains bluish purple; color is not transient.

SR-18 Paraffin-Beeswax Slide Sealing Mixture

Prepared by heating together equal weights of paraffin and beeswax in a beaker or flask immersed in a water bath at 70-80°C. Apply by means of a heated wire or glass rod of small diameter.

SR-19 Shellac - Gum Mastic - Castor Oil Slide Sealing Mixture

- Dissolve about 400 g of high grade flake shellac in 500 ml of absolute alcohol. This may take a week or two with intermittent shaking--less time on a mechanical shaker.
- Filter the solution through porous cloth and bring the final volume to 960 ml with absolute alcohol.
- To each 240 ml of the shellac solution add:
  - 3.7 ml of a saturated solution of gum mastic in absolute alcohol;
  - 3.7 ml of castor oil.
- Mix thoroughly.
- Apply the solution by means of a small brush such as a nail polish applicator

SR-20 Vaspar Sealing Mixture

Equal weights of vaseline and paraffin wax, warmed to liquefy, and thoroughly mixed. Apply with a heated glass rod of small diameter.

SR-21 Plug Poison and Tube Disinfectant (for stock cultures)

95% Ethyl alcohol	950 ml
Glycerine	50 ml
$HgCl_2$	5 g

Add dye as a warning color, Cotton Blue (Poirrier's) or other that is not used for marking plugs for other purposes. Apply to plugs from a dropper bottle. Use precautions in handling.

SR-22 *Hypochlorite Solution (for surface sterilization of seeds, insects, etc.)*

Calcium hypochlorite· A saturated aqueous solution (about 2%) will disinfect the surfaces of small objects within 10-15 min. Specimens can be transferred directly from the hypochlorite to culture media without rinsing because the available chlorine dissipates readily and the calcium is not toxic. The solution must be prepared freshly for each use. Containers of Ca-hypochlorite must be kept tightly stoppered to prevent deterioration of activity.

SR-23 *Disinfectant: 2.5% phenol (for bench tops, incubators, etc.)*

Liquefy crystalline phenol by removing bottle cap and placing container in warm water. When melted, add 10% of its weight of distilled water and agitate. This 90% phenol solution will remain liquid.

To prepare disinfectant, add to a liter of water: 28 ml of 90% phenol, 5 g NaCl, and 5 g of Aerosol wetting agent. Agitate until aerosol dissolves.

SR-24 *Aerosol Solution*

"Aerosol OT" (American Cyanamid) or similar anionic wetting agent; prepared as 1% solution in distilled water. (Ref: Whiffen, A. J. 1946. Mycologia 38:346.)

SR-25 *Agglutination Buffer (for sexual agglutination in yeasts)*

1% $MgSO_4 \cdot 7H_2O$ in 0.01 M Na-acetate.
Adjust to pH 5.5 with acetic acid.
Buffer is not sterilized.

CULTURE MEDIA

Certain general information on culture media is to be found in the introductory chapter of the *Guidebook* (p. 15-19). Where particular media were recommended for carrying out a specified demonstration or investigation, details are included in the section dealing with that study. In addition, a compilation of 100 media has been consolidated in the listings below, and numerous references to these selected media appear in the *Guidebook* as key symbols, usually in parentheses, e.g., (M-17), (M-22a), or (M-6,7).

It will be obvious to the user of the *Guidebook* that many of the media in the following listing do not differ markedly from others in the listing. In these cases the differences may not be critical but reflect, rather, the recommendations of the contributor who submitted that particular study. Many teachers of mycology will have their own favorite media and are encouraged to try those they know best in working out laboratory studies that fit their particular needs. Any such steps to reduce the total number of media used and to simplify procedures are all to the good but can be realized only by actual trial in the individual teaching laboratory.

The following listings can, for convenience, be thought of as falling into several sub-categories. Media 1 through 33 are "natural" media based mostly on materials readily at hand, such as cornmeal, hay, potatoes, or soil. Media 34 through 70 are "semi-synthetic," containing both natural ingredients and defined components. Media 71 through 90 are synthetic or defined media; media 91 through 100 are specialty items developed for growing particular species.

M-1 Brain-heart Infusion Agar

Brain-heart infusion (Difco)	37 g
Distilled water	1000 ml
Agar	15 g

Brain-heart infusion medium can also be used in a diluted form (3.7 g BHI per liter of water).

M-2 *Bread-crumb Agar*

Tapwater	1000 ml
Dried unflavored commercial bread crumbs	100 g
Bacto-Agar	18 g

M-3 *Carrot Wedges*

Wash carrots; cut into long, thick wedges, small enough to fit in 24-mm test tubes. Add 2 ml water, plug and sterilize.

M-4 *Corn Coleoptile-Extract Agar*

Fresh corn coleoptiles	20 g
Distilled water	80 ml
Molten water agar	5 ml

Prepared according to Rowell (1955) by extracting 20 g fresh corn coleoptiles in 80 ml of distilled water by Waring blender fragmentation. The extract is then sterilized by Seitz filtration and 5 ml are added per liter of sterile molten water agar consisting of 16 g of Bacto Nobel's agar per liter of distilled water, cooled to 50°C.

M-5 *Cornmeal Agar*

Yellow cornmeal	40 g
Agar	20 g
Water	1000 ml

Bring water to boil, add cornmeal; simmer 15 min. Filter through three or four layers of cheesecloth into a 1-liter graduated cylinder. Let stand 15 min. Siphon off and retain all but the bottom 200 ml. Bring back to 1 liter volume with water. Add agar. Autoclave to sterilize. (Carmichael, 1957)

M-6 *Cornmeal Agar*

Yellow cornmeal	12.5 g
Agar	6.0 g

Add cornmeal to 300 ml H_2O and heat in water bath at 60°C for one hour with occasional stirring. Filter through No. 1 filter paper. Readjust the volume to 300 ml and add agar.

M-7 *Cornmeal Agar*

Ground yellow corn	50 g
Tapwater	1000 ml

Extract at 80°C for 30 min. Filter through paper or cheesecloth and make the opalescent filtrate to a volume of 1000 ml, using tapwater. Twenty grams of agar are needed to solidify the cornmeal extract. Cornmeal extract agar is available commercially and can be purchased as a dehydrated product.

M-8a *Cornmeal Agar - Half Strength (CM/2)*

Prepare equal quantities of Difco cornmeal agar (without glucose) and Difco 2% plain agar. Mix thoroughly, distribute in tubes and sterilize in the autoclave, 15 min at 15 lbs.

M-8b *Cornmeal Agar - Half Strength 4%*

Difco cornmeal agar	9.5 g
Plain agar	32.5 g
Water	1000.0 ml

M-9 *Cornmeal Yeast Extract Glucose Agar*

This consists of Difco cornmeal-glucose agar made up in accordance with directions on the bottle, but with 0.1% yeast extract added.

M-10 *Dung Extract Agar*

Cow dung (air dry)	200 g
Tapwater	1000 ml
Agar	20 g

Cover the dung with water, boil 20 min, filter with suction through coarse paper (change frequently), make the filtrate to 1000 ml, add agar, and sterilize by autoclaving.

M-11 *Dung Extract Agar*

Horse dung	200 g
Tapwater	1 liter
Agar	20 g

Thoroughly mix dung in tapwater and boil for 15 min; then filter through cheesecloth to remove coarse debris. If a clear gel is required, filtration is carried out hot through diatomaceous earth, using a Buchner funnel. The filtrate, made up to 1 liter with 20 g agar added, is then autoclaved for 15 min at 10 lb.

M-12 *Hay Extract Agar*

Weathered grass (e.g., *Poa* spp., as collected from field, roadside or fence row)	50 g
Tapwater	1000 ml

Combine and autoclave for 20 min at 120°C; then filter.

Infusion filtrate	1000 ml
K_2HPO_4	2 g
Agar	15 g

(Adjust pH to about 6.2; sterilize at 15 lb and 120°C for 20 min.)

For stock cultures and routine cultivation of cellular slime molds.

Dilute Hay Extract Agar for soil isolations; pseudo-plasmodium formation; cultivation of *P. pallidum*. Employed as a broth, dilute hay infusion is very useful for observing feeding habits of myxamoebae in association with *Bacillus megaterium* in slide mounts. Add two volumes tapwater to hay infusion filtrate, add buffer (see above) and 1.5% agar, adjust pH to 6.2 and sterilize.

M-13 *Hay Infusion Agar*

Hay	2.5 g
Tapwater	1 liter

A weak hay infusion agar prepared by steaming in a water bath for 30 min.

Filter the hay off through cheesecloth and add water to restore the volume to 1 liter. Adjust the pH to 7 by adding 1.4 g of $K_2HPO_4 \cdot 3H_2O$; add 15-20 g of agar before autoclaving.

M-14 Lima Bean Agar

Frozen lima beans (or peas)	1 small pkg.
Distilled water to make 1 liter	
Agar	20 g

Boil for 30 min, filter through cloth, save filtrate, add agar. Sterilize.

M-15 Oatmeal Agar

Rolled oats	30 g
Agar	20 g
Water	1000 ml

Cook oatmeal in water for 15-30 min in a container over boiling water (a double boiler is fine). Filter through three or four layers of cheesecloth and bring filtrate back to volume of 1 liter with water. Add agar. Autoclave to sterilize.

M-16 Oatmeal Tomato Agar: Asheshov's A 58 Medium

Heinz Baby Oatmeal	20 g
Tomato paste	20 g
Agar	18 g
Tapwater	1 liter

M-17 Potato-Carrot Agar (PCA)

Potato	20 g (White, peeled)
Carrot	20 g (peeled)
Agar	20 g
Water	1000 ml

Disintegrate the potato and carrot tissue completely in a blender, using a small amount of the water. Add the slurry and agar to remainder of water. Sterilize.

M-18 Potato Glucose Agar

Potato	500 g	(old white, peeled and sliced)
Water	500 ml	

Autoclave together for 20 min. Filter through three or four layers of cheesecloth; squeeze out as much moisture as possible. Bring volume up to 1000 ml with water. (Bottle and sterilize any unused portion for storage.)

Potato extract	100 ml
Glucose	5 g
Agar	20 g
Water	900 ml

Autoclave to sterilize.

M-19 Potato Glucose Agar

Potatoes	200 g
Glucose	20 g
Agar	20 g
Distilled water	1000 ml

Place diced potatoes in water and autoclave 20 min at 15 lb. Filter through cotton, make filtrate to 1000 ml, add glucose and agar, and sterilize by autoclaving.

M-20 Potato Glucose Agar

Flask I -

Distilled water	100 ml
Glucose	20 g
$CaCO_3$	0.2 g
$MgSO_4 \cdot 7H_2O$	0.2 g

Flask II -

Distilled water	400 ml
Agar	15 g

Flask III -

Potatoes (peeled and sliced)	200 g
Distilled water	500 ml

Contents of Flask III brought momentarily to 120°C in an autoclave. Filter through cheesecloth. Bring up to original volume. Simultaneously, the agar in Flask II is melted and the solution in Flask I is heated to boiling. Mix contents of the three flasks. pH not adjusted. Autoclave to sterilize.

M-21 Potato Sucrose Malt Extract Agar

Potatoes	200 g
Sucrose	60 g
Dehydrated malt extract broth	20 g
Peptone	1 g
Agar	20 g
Distilled water	1000 ml

Potatoes are first extracted by boiling in water, the remaining materials added to the filtrate, and the volume brought back to 1000 ml.

M-22a Seawater Serum Agar (1%SSA)

Horse serum (BBL) (add to medium after autoclaving)	1.0% v/v
Agar	1.2% w/v
Seawater	

Modified from Watson and Ordal, 1957.
Optional antibiotics for isolation media: (add to medium after autoclaving)

Streptomycin sulfate	0.25 g/l
Penicillin G	0.25 g/l

M-22b Trypticase Serum Seawater Agar (TS)

Trypticase	0.1% w/v
Horse serum (add to medium after autoclaving)	1.0% v/v
Agar	1.2% w/v
Seawater	

Optional antibiotics for isolation media: (add to medium after autoclaving)

Streptomycin sulfate	0.25 g/l
Penicillin G	0.25 g/l

M-23 Seawater Yeast Extract Glucose Agar

Yeast extract	1 g
Glucose	10 g
Agar	15 g
Seawater	1000 ml

Autoclave to sterilize. (For a formulation of an artificial seawater with about 20 ingredients, see Johnson and Sparrow, 1961, p. 27.)

M-24 Seawater Yeast Extract Glucose Agar (Modified Vishniac's MV)

Glucose	1 g
Gelatin hydrolysate	1 g
Yeast extract	0.1 g
Liver extract (1:20)	0.01 g
Agar	12 g
Seawater	1000 ml

(See Fuller *et al.*, 1964.)
Optional antibiotics for isolation media:
(add to medium after autoclaving)

Streptomycin sulfate	0.25 g/1
Penicillin G	0.25 g/1

M-25 Seawater Yeast Extract Peptone Glucose Agar (YPGS)

Glucose	10 g
Yeast extract	5 g
Peptone	5 g
Agar	12 g
Seawater	1000 ml

M-26 Soil Extract Agar

Garden soil	75 g
Boiling water	500 ml
Agar	20 g

To prepare soil extract agar, approximately 75 g of garden soil is placed within a funnel on filter paper and 500 ml of boiling water is allowed to percolate through it. To this, 20 g of agar (previously dissolved in 500 ml water) are added and the volume made up to 1 liter.

M-27 *Soil Extract Agar*

Field soil	400 g
Tapwater	1 liter

Infuse field soil in tapwater for 30 min at 15 lb and 120°C. Filter through cheesecloth-covered cotton and make clear filtrate up to one liter volume (tapwater). Add:

$NaNO_3$	1 g
Glucose	10 g
Agar	25 g

Adjust pH to 4.5. Sterilize at 12 lb for 20 min with tubes or bottles of agar partially immersed in water to prevent decomposition of agar at low pH. *Remelt only to use.*

M-28 *Steep Agar*

Prepare Czapek's Agar (M-89), omitting sucrose and agar until later. Add 1% by volume of corn steep liquor. Adjust to pH 7.0 with KOH. Add agar and sucrose. Autoclave to sterilize.

M-29 *V-8 Juice Agar*

V-8 juice	200 ml
$CaCO_3$	3 g
Agar	20 g
Water	1000 ml

The calcium carbonate should be in powdered form: it should be maintained in suspension while plates or culture tubes are being filled. Autoclave to sterilize.

M-30 *V-8 Juice Agar*

Take one 6-oz can (180 ml) of V-8 vegetable juice and bring it up to 1 liter with distilled water. Add 20 g agar and 2 g calcium carbonate per liter. The calcium carbonate is needed so the agar will solidify. The final medium is mildly acid, which favors the growth and sporulation of most species. One can prepare one liter of juice and water and divide it equally among four 500-ml flasks, adding the proper amount of agar and calcium carbonate to each flask. The flasks can be placed directly in the autoclave; the agar will melt during sterilization and the flask needs only to be agitated before pouring to assure an even distribution of agar throughout the medium. The oversize flask precludes boiling over while the agar is melting.

M-31 *V-8 Juice Oat Agar*

V-8 juice	50 ml/1
Cream of Oats	40 g/1
Agar	20 g/1

M-32 *V-8 Juice Yeast Agar*

Mix equal parts of commercial canned vegetable juice (V-8) and water. Add 2% dried baker's yeast. Adjust pH to 5.7. Add 2% agar. Melt, tube, plug, sterilize and slant.

M-33 *Wheat Germ Glucose Agar (WG)*

Wheat germ (Kretschmer brand)	15 g
Glucose	5 g
Agar	15 g

Cook 10 min in 1 liter of distilled water; filter, bring volume to 1 liter.

M-34 *Casamino Acids Glucose Agar*

Casamino acids (Difco, vitamin-free)	2.5 g
Glucose	40.0 g
$MgSO_4$	0.1 g
KH_2PO_4	1.8 g
Agar	20.0 g
Distilled water to	1000.0 ml

Adjust pH to 6.8, heat to dissolve ingredients, tube or put in flasks and sterilize.

M-35 Malt Extract Agar

Bacto Malt Extract (Difco)	12.5 g
Bacto-agar (Difco)	20.0 g
Distilled water	1000.0 ml

M-36 Malt Extract Agar

Malt extract (Difco)	10 g
Agar (Difco)	12 g
Water	400 ml

After the solids are dissolved, add the agar and autoclave the medium for 15 min at 121°C.

M-37 Malt Extract Agar

Malt extract	5 g
Agar	2 g
Water	100 ml

Add 0.7 ml 1N HCl per 100 ml of melted medium.

M-38 Malt Extract Agar

Malt extract (Fleischmann's dry diamalt)	10%
Agar	2%

Heat mixture in a boiling water bath until agar is melted. Dispense 5 ml per tube. Plug and sterilize 15 min at 15 lb. Slant tubes.

M-39 Malt Extract Gallic Acid Agar

Bacto malt extract (Difco)	15 g
Bacto agar (Difco)	20 g
Distilled water	1000 ml
Gallic (or tannic) acid	5 g

The medium is prepared by dissolving the agar and malt in 850 ml water in one flask and placing 150 ml water in a second flask and sterilizing both at 20 lb for 15 min. While the 150 ml of water is still hot, the gallic or tannic acid is dissolved in it and the solution is added to the slightly cooled 850 ml malt agar and thoroughly mixed before the mixture is poured into sterile Petri dishes.

M-40 *Malt Extract Glucose Agar*

Agar	15 g
Malt extract	5 g
Glucose	5 g
KH_2PO_4	0.5 g
$MgSO_4 \cdot 7H_2O$	0.5 g
NH_4Cl	0.5 g
Ferric citrate or $FeCl_3$ (1% solution)	0.5 ml
Distilled water	1000 ml

M-41 *Peptone Glucose Agar (Bonner's Medium)*

Glucose	10 g
Peptone	10 g
$Na_2HPO_4 \cdot 12H_2O$	0.96 g
KH_2PO_4	1.45 g
Agar	20 g
Distilled water	1000 ml

pH should be near 6.2; adjust if necessary. Sterilize.

For maximum growth and largest sorocarps in *D. discoideum*, *D. mucoroides*, *D. purpureum*, and *P. violaceum*; migration by *D. discoideum* often somewhat limited.

Lactose is often substituted for glucose when *E. coli* or *A. aerogenes* is used as the bacterial associate.

Half-strength Bonner's Medium--for enhanced migration of *D. discoideum* pseudoplasmodia; excellent for long sorocarps in other large species; not recommended for *P. pallidum*. Composition as above but glucose and peptone at 5 g each/liter.

M-42 *Peptone Glucose Rose Bengal Streptomycin Agar*

Glucose	10 g
Peptone	2 g
KH_2PO_4	0.5 g
$MgSO_4 \cdot 7H_2O$	0.5 g
Rose Bengal	50 mg
Agar	15 g
Water	1000 ml

Streptomycin solution, 8 ml (solution in proportion of 1 g/100 ml sterile distilled H_2O). Prepare medium omitting streptomycin; autoclave to sterilize. Add streptomycin solution aseptically before medium solidifies and distribute it by swirling the container.

M-43 *Peptone Glucose Rose Bengal Streptomycin Agar (Martin's modification)*

Agar	18 g
KH_2PO_4	0.5 g
K_2HPO_4	0.5 g
$MgSO_4 \cdot 7H_2O$	0.5 g
Peptone	5 g
Dextrose	10 g
Yeast extract	0.5 g
Rose Bengal	0.05 g
Streptomycin*	0.03 g

*See M-59 for additional instructions.

M-44 *Peptone Glucose Cycloheximide Chloramphenicol Agar (Sabouraud's with antibiotics)*

Glucose	40 g
Neopeptone	10 g
Agar	20 g
Cycloheximide	0.5 g
Chloramphenicol	0.5 g
Distilled water	1000 ml

M-45 Peptone Lactose Agar (0.1 L-P)

Lactose	1 g
Peptone	1 g
Agar (Difco)	20 g
Distilled water	1000 ml

Sterilize at 15 lb for 20 min.

For cell aggregation in all species of cellular slime molds; sorocarp formation in *Polysphondylium pallidum.*

Glucose may be substituted for lactose and yeast extract for peptone in the above formula to produce other useful combinations- growth is enhanced somewhat on yeast extract.

0.1 G-P/2 and 0.1 G-P/4 Broth or Agar--especially useful for observing feeding habits of myxamoebae with *Bacillus megaterium.* Prepared by diluting 0.1 G-P Broth or Agar with 1 or 3 volumes of non-nutrient broth or agar, respectively.

M-46 Peptone Maltose Glycerine Agar (Sabouraud's)

Peptone	15 g
Maltose	20 g
Glycerine	5 g
Agar	12.5 g
Distilled water	1000 ml

Autoclave to sterilize.

M-47 Peptone Glucose Glycerine Agar (Sabouraud's)

Peptone	15 g
Glucose	20 g
Glycerine	5 g
Agar	12.5 g
Distilled water	1000 ml

M-48 *Peptone Malt Extract Glucose Agar*

Distilled water	1000 ml
Malt extract	20 g
Peptone	1 g
Glucose	20 g
Agar	20-25 g

pH not adjusted

Is particularly recommended for *Aspergillus* and *Penicillium*.

M-49 *Peptone Meat Extract Glucose Agar (Gorodkowa)*

Peptone	1%
Meat extract	1%
Glucose	0.25%
NaCl	0.5%
Agar	2%

Melt, tube, plug, sterilize and slant.

M-50 *Peptone Yeast Autolysate Glucose Broth*

Powdered yeast autolysate (Albimi Corp.)	1%
Proteose peptone (Difco)	1%
Glucose	2%

pH is adjusted to 3.0 with N HCl.
Sterilize the flasks by exposing them to flowing steam (without pressure) for 10 min.

M-51 *Peptone Yeast Extract Glucose (PYG)*

Peptone	1.25 g
Yeast extract	1.25 g
D-glucose	3 g
Distilled water	1 liter

adjust to pH 6.8

PYG is a medium devised for the growth of *Blastocladiella* by E. C. Cantino. It is available commercially as Difco-Cantino PYG broth or agar.

M-52 Peptone Yeast Extract Glucose Agar (PG)

Peptone	5 g
Yeast extract	1 g
Glucose	10 g
K_2HPO_4	0.5 g
$MgSO_4 \cdot 7H_2O$	0.5 g
$FeSO_4 \cdot 7H_2O$	0.05 g
$CaCO_3$	1 g
Agar	15 g
Distilled water	1 liter

The unadjusted pH will be 6.8.

M-53 Peptone Yeast Extract Maltose Agar (Cyathus medium)

Bacto agar	20 g
Maltose	5 g
Glycerine	2 g
Peptone	0.2 g
Asparagine	0.2 g
Yeast extract	2 g
$MgSO_4 \cdot 7H_2O$	0.5 g
$Ca(NO_3)_2$	0.5 g
KH_2PO_4	0.5 g
$FeSO_4$	trace
Distilled water to	1 liter

M-54 Phytone Yeast Extract Glucose Agar

Phytone	10 g
Glucose	40 g
Yeast extract	5 g
Streptomycin	30 mg
Chloramphenicol	50 mg
Agar	20 g
Water	1000 ml

Available in dehydrated form from Baltimore Biological Laboratory. (See also Carmichael, 1962.)

M-55 *Phytone Glucose Rose Bengal Aureomycin Agar*

Glucose	10 g
Phytone	5 g
KH_2PO_4	1 g
$MgSO_4 \cdot 7H_2O$	0.5 g
Rose Bengal	35 mg
Agar	20 g
Water	1000 ml

Aureomycin-HCl solution, 3.5 ml (solution in proportion of 1 g/100 ml sterile distilled water). Prepare medium omitting aureomycin; autoclave to sterilize. Add aureomycin solution aseptically before medium solidifies and distribute it by swirling the container.

M-56 *Tryptone Glucose Agar (Whisler's, modified)*

Tryptone	5 g
Glucose	3 g
Thiamine-HCl	200 µg
KH_2PO_4	280 mg
K_2HPO_4	350 mg
$(NH_4)_2SO_4$	260 mg
$MgCl_2 \cdot 6H_2O$	100 mg
$CaCl_2$	60 mg
Distilled water	1000 ml
Agar	15 g

M-57 *Tryptone Glucose Acetate Agar (Kleyn's Sporulation Medium)*

Bacto-Tryptose (Difco)	0.25%
Glucose	0.062%
NaCl	0.062%
Na acetate·3H O	0.5%
Agar	2%

Melt, tube, plug, sterilize and slant.

M-58 *Tryptone Glucose Yeast Extract Agar (TYG)*

Tryptone	5 g
Yeast extract	5 g
Glucose	1 g
K_2HPO_4	1 g
Agar (Difco)	20 g
Distilled water	1000 ml

pH not adjusted.

For mass cultivation of bacteria to serve as nutrient source for slime molds. Incubate at 37°C for 24 hr; harvest in sterile water, centrifuge, and resuspend to make milk-white suspension.

M-59 *V-8 Juice Oxgall Propionate Streptomycin Chlortetracycline Agar (Papavizas-Davey VDYA)*

	Per liter
Agar	20 g
V-8 juice (filtered)	200 ml
CaCO	3 g
Glucose	5 g
Yeast extract	2 g
Oxgall	5 g
Na propionate	1 g
Streptomycin*	0.03 g
Chlortetracycline*	0.03 g

*These selective antimicrobial agents are prepared as concentrated stock solutions or suspensions (e.g., 100x or 10x) and added to molten agar medium (45-48°C) before pouring plates. All concentrations are based on active ingredients of the chemicals.

M-60 *Yeast Autosylate Glucose Agar*

Yeast autosylate (Albimi)	0.5%
Glucose	5%
Agar	2%

Melt, tube, plug, sterilize and slant.

M-61 Yeast Extract Filter Paper Agar

Yeast extract (Difco)	4 g
Filter paper	12 g
Agar	24 g
Tapwater	1000 ml

In making this medium, the filter paper is dispersed in tapwater, using an electric blender.

M-62 Yeast Extract Glucose Broth

KH_2PO_4	5 g
Glucose	30 g
Yeast extract (Difco)	7 g
Water	1000 ml

After the solids are dissolved, autoclave the medium for 15 min at 120°C. For slants and Petri dishes, add 20 g agar (Difco) to the medium before autoclaving.

M-63a Yeast Extract Glucose Agar

Glucose	10 g
Yeast extract	3 g
K_2HPO_4	2 g
$MgSO_4 \cdot 7H_2O$	0.2 g
Agar	20 g
Distilled water	1 liter

M-63b Yeast Extract Glucose (Emerson's GY5)

Glucose	3 g
Yeast extract (Difco)	1 g
KH_2PO_4	1.4 g
Na_2HPO_4	0.6 g
$MgSO_4 \cdot 7H_2O$	0.1 g
0.04% aqueous bromcresol purple	5 ml
Distilled water	1 liter

M-64 *Yeast Extract Glucose Agar (1-5)*

Glucose	5 g
Yeast extract	1 g
Distilled water	1 liter
Agar	20 g
Microelement solution*	2 ml

pH of this medium need not be adjusted.

*$Fe(NO_3)_3 \cdot 9H_2O$	724 mg
$ZnSO_4 \cdot 7H_2O$	440 mg
$MnSO_4 \cdot 4H_2O$	406 mg

Dissolve in 600 ml distilled water, add sufficient H_2SO_4 to yield a clear solution and make to 1 liter.

M-65 *Yeast Extract Lactose Agar (LY)*

Lactose	1 g
Yeast extract	0.5 g
Bacto-agar	20 g
Distilled water	1 liter

M-66 *Yeast Extract Malt Extract Glucose Agar (1-1-5)*

Glucose	5 g
Yeast extract	1 g
Malt extract	1 g
Distilled water	1 liter
Agar	20 g

M-67 *Yeast Extract Malt Extract Glucose Agar*

Yeast extract	4 g
Malt extract	10 g
Glucose	4 g
Agar	15 g
Distilled water	1000 ml

M-68 *Yeast Extract Malt Extract Sucrose Agar (Osmotic Medium)*

Powdered malt extract	2%
Sucrose	40%
Yeast extract	0.5%
Agar	2.5%

Melt, tube, plug, sterilize and slant.

M-69 *Yeast Extract Malt Extract Peptone Glucose Broth (YM)*

Yeast extract	3 g
Malt extract	3 g
Peptone	3 g
Glucose	10 g
Distilled water	1 liter

YM medium is generally used for cultivation of yeasts. It may be bought on the market already compounded in dry form, or it may be made from the above ingredients. The pH is not adjusted. To make a solid medium, add 25 g of agar. Sterilize by autoclaving.

M-70a *Yeast Extract Soluble Starch Agar (Emerson's YpSs)*

Difco powdered yeast extract	4 g
K_2HPO_4	1 g
$MgSO_4 \cdot 7H_2O$	0.5 g
Difco soluble starch	15 g
Agar	20 g
Water (1/4 tap, 3/4 distilled)	1000 ml

The above medium is most easily prepared by dissolving, separately, the agar and starch in about 400 ml of water and heating to boiling. The inorganic chemicals and the yeast extract should be dissolved separately using portions of the remaining water. The components should be thoroughly mixed and dispensed equally into two 750-ml Erlenmeyer flasks for autoclaving. Standard sterilization procedures may be followed since this medium is not appreciably altered under these conditions. For those laboratories where obtaining multiple chemicals might prove to be a problem, it probably would be more practical to order the above complete medium in the dry form. It may be obtained from supply houses handling Difco products, under the name Bacto-Emerson YpSs Agar, 0739.

M-70b *Yeast Glucose (Emerson's YpG)*

Same as M-70a, but with 20 g glucose in place of starch.

M-71 *Alanine Glucose Agar (Coprinus Fruiting Medium)*

Glucose	10 g
DL alanine	1 g
K_2HPO_4	2 g
$MgSO_4 \cdot 7H_2O$	0.2 g
Thiamin-HCl	500 μg
Agar	20 g
Distilled water	1 liter

Phosphate should be autoclaved separately and added later. The medium should be slightly alkaline: a pH lower than 6.5 retards growth.

M-72 *Amino Acids Melibiose Agar*

Yeast nitrogen base (Difco	0.6%
Melibiose	1%
Agar	2%

Melt, tube, plug, sterilize and slant.

M-73 *Ammonium Nitrate Agar*

Substitute 1.5 g of NH_4NO_3 for casamino acids in the Casamino Acids Glucose Medium (M-34).

M-74 *Ammonium Phosphate Glucose Citrate Nutrient Solution*

$CaCl_2$	0.05 g
NaCl	0.025 g
KH_2PO_4	0.5 g
$(NH_4)_2HPO_4$	0.25 g
$MgSO_4 \cdot 7H_2O$	0.15 g
Ferric citrate, 1% solution	1.2 ml
Thiamin	25 μg
Glucose	2.5 g
Distilled water	1000 ml

M-75 *Ammonium Sulfate Acetate Medium*

The ingredients of this medium are the same as those of the asparagine-acetate medium (M-78), except that 600 mg of $(NH_4)_2SO_4$ are substituted for the asparagine, and the amount of sodium acetate is increased to 13.6 g. Most strains of *Pilobolus kleinii* produce large numbers of asexual reproductive structures on this medium.

M-76 Ammonium Tartrate Glucose Citrate Nutrient Agar

KH_2PO_4	1 g
$MgSO_4 \cdot 7H_2O$	0.5 g
NH_4 tartrate	0.5 g
$ZnSO_4$ (1:500)	0.5 ml
Fe citrate (1%)	0.5 ml
Thiamin	50 µg
Glucose	20 g
Agar	15 g
Distilled water to	1000 ml

M-77 Asparagine Acetate Medium

L-asparagine	7.55 g
Sodium acetate	10 g
KH_2PO_4	0.5 g
K_2HPO_4	0.6 g
$MgSO_4 \cdot 7H_2O$	0.5 g
$CaCl_2$	0.1 g
NaCl	0.1 g
Hemin	10 mg

The medium may be solidified with 2% agar.

M-78 Asparagine Acetate Agar

Distilled water	962 ml
KH_2PO_4	0.5 g
K_2HPO_4	0.6 g
$MgSO_4 \cdot 7H_2O$	0.5 g
NaCl	0.1 g
$CaCl_2$	0.1 g
Asparagine (anhydrous)	7.5 g
Sodium acetate ($CH_3COONa \cdot 3H_2O$)	10 g
Thiamine·HCl	10 mg
Hemin*	10 mg
Micronutrient solution**	1 ml
Agar	20 g

*Dissolve 10 mg hemin in 37.5 ml 0.1 N NaOH, and add the solution to the rest of the medium while swirling it.

**Micronutrient solution:

H_3BO_3	57 mg
$ZnCl_2$	4200 mg
$MnCl_2 \cdot 4H_2O$	72 mg
$NaMoO_4$	42 mg
$CuSO_4 \cdot 5H_2O$	254 mg
$FeCl_3 \cdot 6H_2O$	960 mg
H_2SO_4 (conc.)	1 ml
Distilled water	1000 ml

This medium is recommended for the isolation and maintenance of cultures. Some stock cultures have been maintained on it by weekly transfer of hyphal tips for nine years. Most strains form reproductive structures on it, but not in such abundance as on media containing appropriate concentrations of ammonium ion.

M-79 Asparagine Glucose Agar

KH_2PO_4	0.5 g
K_2HPO_4	0.6 g
$MgSO_4 \cdot 7H_2O$	0.5 g
NaCl	0.1 g
$CaCl_2$	0.1 g
Asparagine	5 g
Glucose	20 g
Agar	20 g
Distilled water	1000 ml

M-80 Asparagine Glucose Agar

Glucose	20 g
Peptone or dl-asparagine	2 g
$MgSO_4 \cdot 7H_2O$	0.5 g
KH_2PO_4	0.46 g
K_2HPO_4	1 g
Thiamine·HCl	120 µg
Agar	20 g
Distilled water	1 liter

M-81 Asparagine Glucose Agar

Glucose	40 g
Asparagine	2 g
KH_2PO_4	0.5 g
$MgSO_4 \cdot 7H_2O$	0.25 g
Thiamine chloride	0.5 mg
Agar	15 g
Distilled water	1000 ml

M-82 Glucose Mineral Agar - Basal Synthetic Mdeium

Sugar (usually glucose)	Variable, usually 3-20 g
Nitrogen source	Usually 1-2 g
KH_2PO_4	1 g
$MgSO_4 \cdot 7H_2O$	0.5 g
Fe, Zn, Mn (provided by 2 ml of stock solution; see medium #64)	
Ca	10 mg
Thiamine	100 µg
Biotin	5 µg
Pyridoxine	100 µg (required by only a few fungi)
Distilled water	1 liter
Agar (if desired)	20 g

pH adjusted to near 6.0 - 6.5

If used as a liquid for obtaining dry weights, 250-ml Erlenmeyer flasks, each contining 25 ml of medium and capped with 25-ml beakers (or plugged with cotton) are suggested. For most purposes agar media are more convenient and can be used in Petri dishes, each with about 20 ml.

M-83 Glucose Mineral Agar (Czapek-Dox)

As M-89. but with 2% glucose instead of 3% sucrose, is a satisfactory minimal medium for *Aspergillus nidulans*.

M-84 Glucose Salts Agar

Dissolve 11.7 g of yeast carbon base (Difco) in 900 ml of distilled water. Adjust the pH to 7.0 with 1N KOH and bring the volume to 1 liter. Add 20 g of agar, autoclave and pour into Petri dishes. Yeast carbon base is a commercially prepared mixture of salts, trace elements, vitamins, and glucose but does not contain a nitrogen source.

M-85 Glucose Salts Conjugation Medium

KH_2PO_4 at pH 5.5	0.01 M
$MgSO_4 \cdot 7H_2O$	0.1%
Glucose	0.5%

The conjugation medium is not sterilized, but the glucose can be prepared in advance by autoclaving a 5% solution for 15 min at 120°C and then diluting 1/10 into the medium.

M-86 Glucose Salts Agar Plus Nitrate

Yeast carbon base (Difco)	11.7 g
KNO_3	3.5 g
Agar	20 g
Distilled water	1 liter

M-87 Mineral Agar

NH_4NO_3	3 g
KH_2PO_4	1 g
$MgSO_4 \cdot 7H_2O$	0.5 g
KCl	0.5 g
Agar	15 g
Water	1000 ml

Autoclave to sterilize. Use with carbon sources that have been sterilized separately (grass cornstalks, cotton thread). (See also Farrow, 1954.)

M-88 Proline Sucrose Agar

Sucrose	6 g
Proline	2.7 g
K_2HPO_4	1.3 g
KH_2PO_4	1 g
KCl	0.5 g
$MgSO_4 \cdot 7H_2O$	0.5 g
$FeSO_4 \cdot 7H_2O$	10 mg
$ZnSO_4 \cdot 7H_2O$	2 mg
$MnSO_4 \cdot 4H_2O$	1.6 mg
Agar	20 g
Distilled water	1000 ml

Autoclave to sterilize.

M-89 Sucrose Mineral Agar (Czapek-Dox)

$NaNO_3$	3 g
K_2HPO_4	1 g
$MgSO_4 \cdot 7H_2O$	0.5 g
KCl	0.5 g
$FeSO_4 \cdot 7H_2O$	0.1 g (1 ml of 1% aqueous solution)
Sucrose	30 g
Water	1000 ml
Agar	20 g

Dissolve all salts in water, adding K_2HPO_4 after all other salts have dissolved. To reduce caramelization, the sugar is added just prior to final sterilization Sterilize at 15 lb and 120°C.

Czapek solution agar with 20%, 30%, or 40% sucrose. As above except 200 g, 300 g, or 400 g sucrose per liter. Sterilize as above.

M-90 Water Agar (Plain Agar, Nonnutrient Agar)

Agar (Difco)	20 g
Distilled water	1000 ml

Medium on which to streak or spread pregrown bacteria as nutrient source for myxamoebae. Slime mold growth proportional to bacteria added.

M-91 *Labyrinthula Zoosporulation Medium (GA)*

NaCl	2.4 g
$MgSO_4 \cdot 7H_2O$	0.8 g
KCl	70 mg
K_2HPO_4	43 mg
$CaCl_2$	47 mg
NaH glutamate	0.2 g
Glucose	0.4 g
Thiamine HCl	20 μg
Biotin	0.1 μg
B_{12}	0.1 μg
Trace metals	
NaEDTA	5 mg
$FeSO_4 \cdot 7H_2O$	0.25 mg
$ZnSO_4 \cdot 7H_2O$	0.09 mg
$MnSO_4 \cdot 4H_2O$	0.03 mg
$CoSO_4 \cdot 7H_2O$	9.5 μg
$CuSO_4 \cdot 5H_2O$	0.8 μg
H_3BO_3	11.2 μg
$Na_2MoO_4 \cdot 2H_2O$	3.7 μg
Glass-distilled water to	1000 ml

pH adjusted to 7.4.

M-92 M_3 *Chytrid Agar*

Difco cornmeal agar	17 g
Glucose	5 g
Soluble starch	5 g
Peptone	1 g
Yeast extract	1 g
Glass-distilled water	1000 ml

M-93 *Allomyces Development Media*

a) Mineral Solution (BSM)

An inorganic salts solution composed of:

1×10^{-3}M $MgSO_4$
1×10^{-3}M $CaCl_2$
2.5×10^{-3}M NH_4NO_3
8×10^{-3}M KCl
5×10^{-3}M KH_2PO_4-K_2HPO_4
at pH 6.6

Just before use, it is filtered through a 1.2µ Millipore filter (Type RA Millipore Filter Corp., Bedford, Mass.) to reduce the background count (Murphy and Lovett, 1966).

b) Glucose Salts Medium (Synthetic Medium B)

Thiamine HCl	0.15 mg/liter
Dl methionine	0.1 g/liter
$MgSO_4$	--
$MgCl_2$	0.0005 M
K_2HPO_4	0.005 M
KH_2PO_4	0.005 M
$(NH_4)_2HPO_4$	0.005 M
$CaCl_2$	0.0005 M
Difco yeast extract	--
$MnCl_2$	0.5 ppm Mn
$ZnSO_4$	0.1 ppm Zn
$CuSO_4$	0.1 ppm Cu
$FeCl_3$	1.0 ppm Fe
$(NH_4)_6Mo_7O_{24}$	0.2 ppm Mo
$CoCl_3$	0.2 ppm Co
H_3BO_3	0.5 ppm B
KOH	--
Glucose	5.0 g/liter

Glucose autoclaved separately. pH is automatically 7.0. Solution of $CaCl_2$ and $MgCl_2$ separately autoclaved, composited with separately autoclaved glucose solution, and added aseptically to rest of medium.

Derived from Emerson's YpSs medium. (Emerson, 1941)

c) DS (dilute salts) Solution

1. (Machlis)

KH_2PO_4	0.001 M
$MgCl_2$	0.0001 M
$CaCl_2$	0.00002 M

KOH to pH 7.0

2. (Murphy and Lovett)

KH_2PO_4	0.0005 M
K_2HPO_4	0.0005 M
$(NH_4)_2HPO_4$	0.0005 M
$MgCl_2$	0.00005 M
$CaCl_2$	0.00005 M

M-94 Coprinus Genetics Media

a) Minimal Medium:

Glucose	20 g	
Asparagine	2 g	
Salt solution*	25 ml	
Thiamine	1 mg	
Trace element solution**	40 ml	(omitted in solid media)
Distilled water to	1 liter	

*Salt Solution

NH_4 tartrate	10 g
KH_2PO_4	20 g
Na_2HPO_4	45 g
Na_2SO_4	5.6 g
Distilled water to	500 ml

**Trace Element Solution

$CaCl_2$	1 g
$MgCl_2$	4.1 g
Ferric citrate	531 mg
Citric acid	531 mg
$MnSO_4 \cdot 4H_2O$	443 mg
$ZnSO_4 \cdot 7H_2O$	405 mg
Distilled water to	400 ml

b) Complete Medium:

Minimal medium components (above) plus:

Yeast extract	0.75 g	
Hydrolyzed casein	0.75 g	
Malt extract	0.60 g	
Hydrolyzed nucleic acid	1.25 ml	(see M-100f)
Distilled water to	1 liter	

c) Natural Fruiting Medium:

Moistened horse dung is sterilized by autoclaving at 10-15 p.s.i. for 30 min. The dung is most conveniently prepared in widemouth milk bottles 6-8 in high, capped with aluminum foil.

d) Synthetic Fruiting Medium:

Casselton and Casselton (1966) report that wild type dikaryons will fruit on minimal medium containing from 2×10^{-4}M to 2×10^{-3}M magnesium sulphate. The optimum concentration appears to be 1×10^{-3}M.

M-95 Discomycete Development Media

a) Ascobolus Fruiting Medium

Yeast extract	3 g
Bacto agar	25 g
Distilled water	1000 ml
Filter paper	1 disk (9 cm)

This medium is prepared in the following way: Four holes (windows) are made in each disk of filter paper at equidistant points about 1 cm from the edge. The disks are sterilized in Petri dishes (50-100 per dish) in the autoclave and then placed into empty, sterile Petri dishes (one per dish). The yeast extract-agar solution is also sterilized by autoclaving and dispensed into the dishes containing the filter paper disks (ca. 25 ml/dish).

b) Pyronema Media

KH_2PO_4	0.05 g
NH_4NO_3	0.05 g
$MgSO_4$	0.02 g
$FePO_4$	0.001 g
Distilled water	1000 ml

Medium A: Solidify 700 ml of the above mixture with 14 g agar. Autoclave.

Medium B: Add 6 g inulin to the remaining 300 ml of the original mixture, solidify with 6 g agar. Autoclave.

c) Jiffy-7 Pellet Infusion Agar (*Peziza*)

Jiffy-7 Pellet infusion agar is prepared by homogenizing three Jiffy-7 Pellets per liter plus 20 g agar. The pH should be adjusted to 6.5 prior to autoclaving.

M-96 Phytophthora and Pythium Media

	Per liter
Dehydrated cornmeal agar (Difco)	17 g
Pimaricin*	0.01 g
Vanocomycin*	0.2 g
Pentachloronitrobenzene (PCNB)*	0.1 g

The polyene antifungal antibiotic, pimaricin, inhibits the growth and spore germination of almost all fungi except the pythiaceous members and a few others (e.g., *Mortierella*). Incorporation of PCNB, also nontoxic to most *Phytophthora* and *Pythium* spp., further enhances the control of undesired fungi. Vancomycin inhibits virtually all soil actinomycetes and effectively suppresses most soil bacteria. Other antibacterial antibiotics (e.g., chloramphenicol, chlortetracycline, oxytetracycline, etc.) that are commonly used in many fungal isolation media, are toxic to most species of *Phytophthora* and *Pythium*. These chemicals, therefore, are not suitable ingredients for controlling bacteria and actinomycetes in a *Phytophthora* or *Pythium* isolation medium.

The medium used for quantitative studies should be freshly prepared. Since pimaricin is sensitive to light, isolation plates should be incubated in darkness to prevent loss of potency due to photoinactivation. Higher concentrations of pimaricin should be avoided as the antibiotic becomes inhibitory to spore germination of many *Phytophthora* and *Pythium* species if concentrations of 0.025 g/liter or higher are used.

*See M-59 for further details.

M-97 Rhizoctonia solani Medium

	Per liter
Agar	20 g
K_2HPO_4	1 g
$MgSO_4 \cdot 7H_2O$	0.5 g
KCl	0.5 g
$FeSO_4 \cdot 7H_2O$	0.01 g
$NaNO_2$	0.2 g
Gallic acid*	0.4 g
Streptomycin*	0.95 g
Chloramphenicol*	0.05 g
Na p-(dimethylamino) benzenediazosulfonate (Dexon)*	0.063 g

In this medium streptomycin and chloramphenicol are the antibacterial antibiotics, and Dexon controls the fast-growing pythiaceous fungi. Gallic acid and sodium nitrite serve as selective carbon and nitrogen sources, respectively, and are stimulatory to the growth of *R. solani*. In addition, gallic acid at the concentration used is inhibitory to many undesired fungi.

Because of low concentrations of resistant propagules of *R. solani* in soil, the conventional soil dilution plate method is often not satisfactory for enumerating *Rhizoctonia* populations. Semiquantitative data can be obtained by simply plating out soil clumps of 100-200 mg directly on the surface of solidified selective agar medium.

*See M-59 for further details.

M-98 Schizophyllum Genetics Media

a) Minimal (MM)

	Per liter
KH_2PO_4	0.46 g
K_2HPO_4	1 g
$MgSO_4 \cdot 7H_2O$	0.5 g
Glucose	20 g
dl-asparagine	20 g
Thiamine HCl	100 µg
Agar (BBL or Bacto-Noble)	20 g

b) Complete (CM)

	Per liter
KH_2PO_4	0.46 g
K_2HPO_4	1 g
$MgSO_4 \cdot 7H_2O$	0.5 g
Glucose	20 g
Bacto-peptone	2 g
Agar (BBL or Bacto-Noble)	20 g

Combine dry ingredients in flask of suitable size and add distilled water. Swirl to mix, then cap and autoclave for 15 min at 15 p.s.i. Pour ca. 50 plates/liter while medium is hot. For slants: autoclave, dispense, plug, re-autoclave and slant.

c) Complete plus Yeast Extract (CYM)

Complete medium with 2-5 g/liter yeast extract added.

M-99 Thielaviopsis basicola Medium

	Per liter
Agar	17 g
KH_2PO_4	0.5 g
K_2HPO_4	0.5 g
$MgSO_4 \cdot 7H_2O$	0.5 g
Peptone	0.5 g
Glucose	10 g
Yeast extract	0.5 g
Rose bengal	0.05 g
Streptomycin*	0.03 g
Nystatin*	0.03 g
Pentachloronitrobenzene (PCNB)*	0.5 g

This medium is essentially a combination of Papavizas' medium (1964) and modified Martin's medium (1950). The nystatin and PCNB at the concentrations used are noninhibitory to the growth and spore germination of *T. basicola*, but effectively eliminate, or restrict the size of, undesired fungal colonies in the medium. Streptomycin in combination with rose bengal effectively controls bacteria and actinomycetes. An additional antibiotic, chlortetracycline at 0.002 g/liter, can be used to further enhance bacterial control.

*See M-59 for further details.

M-100 Ustilago Genetics Media

a) Minimal Medium

Glucose	10 g
KNO_3	3 g
Salt solution(c)	62.5 ml
Distilled water	1 liter

b) Complete Medium

Glucose	10 g
Hydrolyzed casein	2.5 g
Hydrolyzed nucleic acid (f)	5 g
Vitamin solution (e)	10 ml
Yeast extract	1 g
NH_4NO_3	1.5 g
Salt solution (c)	62.5 ml
Distilled water to	1 liter

c) Salt Solution

KH_2PO_4	16 g
Na_2SO_4	4 g
KCl	8 g
$MgSO_4 \cdot 7H_2O$	2 g
$CaCl_2$	1 g
Trace elements (d)	8 ml
Distilled water to	1 liter

d) Trace Element Solution

H_3BO_3	30 mg
$MnCl_2 \cdot 4H_2O$	70 mg
$ZnCl_2$	200 mg
$Na_2MoO_4 \cdot 2H_2O$	20 mg
$FeCl_3 \cdot 6H_2O$	50 mg
$CuSO_4 \cdot 5H_2O$	200 mg
Distilled water	500 ml

e) Vitamin Solution

Thiamine (aneurin)	100 mg
Riboflavin	50 mg
Pyridoxin	50 mg
Calcium pantothenate	200 mg
Nicotinic acid (niacin)	200 mg
Choline chloride	200 mg
Inositol	400 mg
Distilled water to	1 liter

f) Nucleic Acid Hydrolysate

Add 1 g yeast nucleic acid and 1 g thymus nucleic acid in 15 ml N sodium hydroxide, and 1 g of each nucleic acid in 15 ml N hydrochloric acid; heat the two mixtures in the autoclave at 15 lb for 10 min; then mix the hydrolysates, adjust pH to 6.0, filter hot, add distilled water to give a final volume of 40 ml. Store in the deep freeze, or at 4°C with 1 ml chloroform.

Films and Film Loops

There are in existence a large number of mycological films, many of which are neither sufficiently accurate nor of sufficiently high technical quality to be useful in college teaching laboratories. At the same time, the following listing makes no pretense of being complete, nor is there any implied quality evaluation, although it is intended as a representative list of useful films. Several general catalogs are available:

- Institut für den Wissenschaftlichen Film, 34 Göttingen, Nonnenstieg 72, BRD.
- Films for Universities, British Universities Film Council, 72 Dean Street, London W.1, U.K. Price £1.75. (Classification according to Universal Decimal Classification system.)
- Film Library Catalogue, The Royal Microscopical Society, Clarendon House, Cornmarket Street, Oxford, U.K.

In the list below, information to the extent it is available is given in the following order: Title, author, reference or catalog number, and source. Where the type of film is known (color or black-and-white, sound or silent, and running-time), this information is given in parentheses following the title.

- Slime molds I: Life cycle (color, sound, 30 min); J. L. Koevenig; U-5519; Bureau of Audio-Visual Instruction, Extension Division, State University of Iowa.

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- Slime molds II: Collection, cultivation and use (color, sound, 19 min); J. L. Koevenig; U-5519; Bureau of Audio-Visual Instruction, Extension Division, State University of Iowa.
- Slime molds III: Identification (color, sound, 24 min); J. L. Koevenig; U-5520; Bureau of Audio-Visual Instruction, Extension Division, State University of Iowa.
- *Physarum polycephalum* (b/w, silent, 25 min); E. Guttes, S. Guttes, and H. P. Rusch; John Ott Pictures, Inc., Attn. Mr. John Ott, 1873 Hillview St., Sarasota, Fla. 33579.
- Entwicklung von *Didymium nigripes* (Myxomycetes) Amöben-Phase (b/w, optical sound, 8 min); N. S. Kerr; C-1044; Institut für den Wissenschaftlichen Film, 34 Göttingen, Nonnenstieg 72, BRD.
- Entwicklung von *Didymium nigripes* (Myxomycetes) plasmodium-phase (b/w, optical sound, 9 min); N. S. Kerr; C-1045; Institut für den Wissenschaftlichen Film, 34 Göttingen, Nonnenstieg 72, BRD.
- *Echinostelium minutum* (Myxomycetes) amoebal phase (b/w, silent, 10½ min); E. E. Haskins; E-1816; Institut für den Wissenschaftlichen Film, 34 Göttingen, Nonnenstieg 72, BRD.
- *Echinostelium minutum* (Myxomycetes) plasmodial phase (b/w, silent, 10½ min); E. E. Haskins; E-1817; Institut für den Wissenschaftlichen Film, 34 Göttingen, Nonnenstieg 72, BRD.
- Fruiting in *Dictyostelium* (silent, 10 min); J. T. Bonner; Department of Biology, Princeton University, Princeton, N.J.
- The use of *Physarum polycephalum* in studies on growth and differentiation; H. P. Rusch; Dr. H. P. Rusch, McArdle Laboratory, University of Wisconsin, Madison, Wis., or John Ott Pictures, Inc., 1873 Hillview St., Sarasota, Fla. 33579.
- Entwicklung von *Dictyostelium* (b/w, magnetic sound, 14½ min); G. Gerisch; C-876; Institut für den Wissenschaftlichen Film, 34 Göttingen, Nonnenstieg 72, BRD.
- *Labyrinthula coenocystis* (Protomyxidea), Bewegung und Fortpflanzung (b/w, silent, 7 min); K. G. Grell; E-1172; Institut für den Wissenschaftlichen Film, 34 Göttingen, Nonnenstieg 72, BRD.

- Motility in *Labyrinthula* (b/w, silent, 11 min); D. Porter; Educational Services, University of Washington Press, Seattle, Wash. 98105.
- A marine slime mold, *Labyrinthula* (16 mm, sound); D. Porter; Education Development Center, Film Studio, 39 Chapel St., Newton, Mass. 02160.
- *Polymyxa betae* (Plasmodiophoraceae): Vegetative vermehrung in Wurzelhaar der Zuckerrübe (b/w, silent, 11 min); B. Keskin and W. H. Fuchs; E-1001; Institut für den Wissenschaftlichen Film, 34 Göttingen, Nonnenstieg 72, BRD.
- Phycomycetes: Sporulation bei Thraustochytriaceae (b/w, optical sound, 8 min); A. Gaertner; D-1078; Institut für den Wissenschaftlichen Film, 34 Göttingen, Nonnenstieg 72, BRD.
- *Thraustochytrium kinnei* (Thraustochytriaceae): Vegetative Entwicklung (b/w, silent, 10½ min); A. Gaertner; E-1664; Institut für den Wissenschaftlichen Film, 34 Göttingen, Nonnenstieg 72, BRD.
- Reproduction in *Monoblepharis* (b/w, silent, 20 min); H. C. Whisler; Dr. H. C. Whisler, Department of Botany, University of Washington, Seattle, Wash. 98105.
- Syngamy and alternation of generations in *Allomyces*, a water mold (b/w, silent, 20 min); R. Emerson; Rental--Media Center, University Extension, University of California, Berkeley, Calif. 94720; or British Film Institute, 42/43, Lower Marsh, London S.E.1, U.K.
  Sale--Audio-Visual Department, School of Education, University of the Pacific, Stockton, Calif. 95204.
- Developmental pathways in *Blastocladiella emersonii* (color, 16 mm, sound, 15 min); E. C. Cantino.
- Motility of *Blastocladiella* spores and *Allomyces* gametes (1/40 natural rate) (b/w, silent 5 min); M. E. J. Holwill; Dr. M. E. J. Holwill, Department of Physics, Queen Elizabeth College, London W.8, U.K.
- Zoosporangium discharge in *Rhizidomyces* (b/w, silent, 10 min); M. S. Fuller and I. K. Ross; Prof. M. S. Fuller, Department of Botany, University of Georgia, Athens, Ga. 30601.
- Sporangial discharge of *Pythium middletonii* (b/w, silent, 8 min); J. Webster; Prof. J. Webster,

Department of Biological Sciences, University, Exeter, Devon Ex4 4PS.

- Growth and asexual reproduction in *Saprolegnia ferax* (b/w, silent, 22 min); A. D. Greenwood; Mr. A. D. Greenwood, Department of Botany, Imperial College, London S.W.7, U.K.
- Asexuelle Vermehrung von *Saprolegnia mixta* (Saprolegniaceae) (b/w, magnetic sound, 9 min); A. Gaertner; C-1080; Institut für den Wissenschaftlichen Film, 34 Göttingen, Nonnenstieg 72, BRD.
- Sexuelle Fortpflanzung von *Saprolegnia mixta* (Saprolegniaceae) (b/w, optical sound, 7 min); A. Gaertner; C-1079; Institut für den Wissenschaftlichen Film, 34 Göttingen, Nonnenstieg 72, BRD.
- *Lagenisma coscinodisci* (Lagenidiales): Vegetative Vermehrung in der Kieselalge *Coscinodiscus granii* (b/w, silent, 16 min); G. Drebes; E-1398; Institut für den Wissenschaftlichen Film, 34 Göttingen, Nonnenstieg 72, BRD.
- Lebenzyklus von *Flammulina velutipes* (Agaricales) (b/w, magnetic sound, 8½ min); G. Eger; C-1083; Institut für den Wissenschaftlichen Film, 34 Göttingen, Nonnenstieg 72, BRD.
- Der Halbschalentest-Zur Biologie des Kulturechampignons *Agaricus bisporus* Leg (Sing) (color, magnetic sound, 8½ min); G. Eger; W-688; Institut für den Wissenschaftlichen Film, 34 Göttingen, Nonnenstieg 72, BRD.
- The cultivation of Japanese edible fungi (color, optical sound {English}, 50 min); K. Mori; Prof. K. Mori, Mori Mushroom Research Institute, 8-1, Hirai-Cho, Kiryu-Shi, Gumma-Keyn, Japan.
- Cultivation of shii-take mushroom (*Lentinus edodes* {Berk} Singer) in Japan (color, optical sound {Japanese}, 30 min); N. Hiratsuka; Prof. N. Hiratsuka, Wakaba - cho-1-1, Chofu-shi, Tokyo, Japan 182.
- Conidium ontogeny in Hyphomycetes; G. T. Cole and W. B. Kendrick; Dr. W. B. Kendrick, Department of Biology, University of Waterloo, Ontario.
- The biology of *Atta*, the ants which grow mushrooms (color, sound, 22 min); S-62; Society for French American Cultural Services and Educational Aid,

972 Fifth Avenue, New York, N.Y. 10021, or Service du Film de Recherche Scientifique, Paris.

- Champignons predateurs de nematodes: Three parts (silent, French subtitles, 45 min).
- Commercial mushroom culture; American Mushroom Institute, P.O. Box 373, Kennett Square, Pa. 19348.
- Basidiomycetes I-IV (*Polystictus* filmed by Girbardt); Deutsches Institut für Film, Bild und Ton, Krausenstr. 9-10, 108 Berlin, DDR.
- Carnivorous fungi (b/w, silent, 8 min); M. Higgins and D. Pramer; Rental--Committee on Visual Aids of the American Society for Microbiology, 1913 I St., N.W., Washington, D.C. 20006; Sale--Prof. D. Pramer, Director of Biological Sciences, Rutgers University, New Brunswick, N.J. 08903.
- Life of the molds (color and b/w, 21 min); McGraw-Hill Text Films, 330 West 42nd St., New York, N.Y.
- The diagnosis and management of cutaneous blastomycosis (Gilchrist's disease) (b/w, sound); American Society of Microbiology, 1913 I St., N.W., Washington, D.C.
- Mississippi Valley disease, histoplasmosis (b/w, sound, 30 min); American Society of Microbiology, 1913 I St., N.W., Washington, D.C. 20006.
- North American blastomycosis (b/w, sound); The Department of Medical Communications, University of Kansas Medical Center, Rainbow Blvd. at 39th St., Kansas City, Kans. 66103.
- An epidemic of histoplasmosis (b/w, sound, 17 min); M-534; The Department of Medical Communications, University of Kansas Medical Center, Rainbow Blvd. at 39th St., Kansas City, Kans. 66103.
- Coccidioidomycosis: Its epidemiologic and clinical aspects (color, sound, 19 min); M-175; National Medical Audiovisual Center (Annex), Station K, Atlanta, Ga. 30324.
- Histoplasmosis, Mason City (color, sound, 16 min); M-1228; Rental--National Medical Audiovisual Center (Annex), Station K, Atlanta, Ga. 30324; Sale--Du Art Film Laboratories, Inc., 245 W. 55th St., New York, N.Y. 10019.
- Griseofulvin: Treatment of superficial fungus infection (color, sound); SS-221; Schering

Professional Film Library, c/o The Association Films, Inc., 561 Hillgrove Ave., LaGrange, Ill. 60525.

- Fungus infection of the foot (color, sound); SS-136; Schering Professional Film Library, c/o The Association Films, Inc., 561 Hillgrove Ave., LaGrange, Ill. 60525.

In addition to films, *per se*, some instructional material is available as film-strips. The list below, for example, contains a number of 35 mm strips, mostly in the area of medical mycology, that are available from the National Archives and Records Service, National Audiovisual Center, Washington, D.C. 20409. Each is accompanied by a 16-inch, 33-1/3 sound disc.

- A mycological slide culture technique (F-24).
- Common saprophytic fungi (F-446).
- Laboratory diagnosis of ringworm in animals. Part I. *Microsporum* infections (F-221).
- Laboratory diagnosis of ringworm in animals. Part 2. *Trichophyton* infections (F-221a).
- Laboratory diagnosis of Tinea Capitis in children: *Microsporum* infections (5-127).
- Laboratory diagnosis of *Trichophyton* infections. Part 1. Ectothrix infections of beard and scalp (F-94a).
- Laboratory diagnosis of *Trichophyton* infections. Part 2. Endothrix infections of the scalp (F-94b).
- *Blastomyces dermatitidis* (F-116a).
- *Coccidioides immitis* (F-116b).

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